IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

KAPLAN, AARON

Serial No.: 09/887,038

Filed: 25 June, 2001

For: ENHANCED INORGANIC CARBON FIXATION BY PHOTOSYNTHETIC PLANTS

Group Art Unit: 1638

Attorney Docket: 01/22171

Examiner: KUBELIK, ANNE R. PhD.

Commissioner for Patents
PO Box 1450
Arlington, VA 22313-1450

DECLARATION OF PROF AARON KAPLAN UNDER 37 CFR 1.132

I am presently employed as a full professor at the Hebrew University of Jerusalem, serving as the Chairman of the Department of Plant Science, where I am engaged in research and teaching. I also serve as the Director of the Minerva center for arid ecosystem research and a member of the Minerva Research Center on photosynthesis under stress.

I received my Ph.D. degree from the Hebrew University of Jerusalem in 1975, worked as a post-doctoral fellow in the Carnegie Institution at Stanford, California. During my post-doctoral studies, we discovered the CO_2 concentrating mechanism which operates in many photosynthetic microorganisms to raise the internal CO_2 concentration at the site of carboxylation. Over the years, I have served as a visiting Professor at Tokyo, Konstanz and Nagoya Universities. A Curriculum Vitae is appended.

My research focuses on Molecular mechanisms that drive ecological processes and on factors rate-limiting photosynthesis and growth of photosynthetic organisms. Since the beginning of my career, I have published over 100 scientific articles in highly regarded journals including PNAS, Current Biology, Plant Cell, JBC, Plant

Physiology and others, and have authored several invited reviews for, among others, Annu. Rev. Plant Physiology Plant Mol. Biol. Over the years I have presented my studies in many invited plenary, keynote and symposia lectures in international scientific conferences. I frequently serve as a reviewer for Nature, Science, PNAS, JBC, Plant Cell, Plant Physiology and others and for various granting agencies including NSF, USDA and DOE.

I am a member of several scientific societies including the American Society of Plant Biologists, The European society of Microbiologists and others.

I am a coinventor of the subject matter claimed in the above-referenced U.S. patent application.

I have read the Official actions issued with respect to the above-identified application.

In a telephone interview of July 24, 2003, the Examiner requested clarification regarding the relevance of the reference cited in the previous communication of May 28, 2002 (Omata et al, PNAS 1999, 96:13571-76), from which the Examiner concluded that cmpA serve as the bicarbonate transporter in cyanobacteria and that the "enzymatic activity of the gene of SEQ ID NO:2 (*ictB* gene) remains in question". Such clarification is provided hereinbelow.

Firstly, regarding the role of bicarbonate transporters in Ci acquisition systems- I wish to point out that it is now well established that the ability to actively concentrate CO_2, against a gradient, results from the activity of at least 4 separate protein systems. This emerged from collaborative studies by Ogawa and myself : (Shibata, M., Katoh, H., Sonoda, M., Ohkawa, H., Shimoyama, M., Fukuzawa, H., Kaplan, A. and T. Ogawa (2002) Genes Essential to Sodium-Dependent Bicarbonate Transport in Cyanobacteria: Function and Phylogenetic analysis. JBC 277: 18658-18664; and Shibata, M., Ohkawa, H., Kaneko, T., Fukuzawa, H., Tabata, S., Kaplan, A. and T. Ogawa (2001) Distinct constitutive and low-CO_2-induced CO_2 uptake systems in cyanobacteria: Novel genes involved and their phylogenetic relationship with homologous genes in other organisms. Proc. Natl. Acad. Sci. USA 98: 11789-11794).

Those were summarized in a recent invited review (Ogawa and Kaplan, Photosynthesis Research, in press, preprint enclosed). There are at least two CO_2

uptake systems (an induced and a constitutive system), and two HCO_3^- transporter systems, the *cmp*A-D system, and the SbtA are active in cyanobacteria (see Table 2, page 4). These studies clearly indicated the limited role of the cmpA-D system, if any, in cyanobacteria since inactivation of this system in *Synechocystis* PCC 6803 (or *Synechococcus* PCC 7942) had little effect on either bicarbonate uptake or ability to grow under low CO_2 conditions (see page 4, left column, paragraph 1).

Thus, it is evident that the *cmp*A-D HCO_3^- transporter system plays only a minor role in *Synechocystis* PCC 6803 growth, and that other, more important HCO_3^- transporter systems operate in this, and other, photosynthetic organisms. Indeed, we have recently shown that in a mutant of *Synechocystis* PCC 6803 where we inactivated CO_2 uptake and HCO_3^- uptake by the sbtA system the ability to grow under low CO_2 conditions was lost despite the fact that the cmpA-D operon was not affected. Inactivation of this operon in this mutant had no effect on growth (Shibata et al, J Biol Chem 2002, 277:18658-64, enclosed; see Figs. 1A and 1B).

In a very recent study, yet unpublished, we found that exposing a *Synechocystis* PCC 6803 mutant in which the two systems for CO_2 uptake and cmpA-D and sbtA systems for HCO_3^- uptake were inactivated, to salinity restored HCO_3^- uptake capability and enabled the cells to grow under low CO_2, due to reactivation of the ictB system. This data provided additional evidence for the role of ictB in HCO_3^- uptake in cyanobacteria and for the presence of multiple pathways for Ci acquisition in these organisms, emphasizing the fallacy of Omata's conclusions.

Further, the Examiner has stated that "enzymatic activity of the gene of SEQ ID NO:2 (*ictB* gene) remains in question". It is my strong opinion that the bicarbonate transporter activity of the *ictB* gene product is amply demonstrated in the instant specification. For example, a *Synechococcus* PCC 7942 mutant where *ictB* was inactivated (mutant IL-2), was severely depressed in inorganic carbon uptake, compared with wild type (Figure 4a and 4b). The impaired HCO_3^- uptake was especially notable in low CO_2 conditions (see Table 1, page 47). In another example, transgenic plants expressing the *ictB* gene demonstrated superior photosynthetic rate, compared to wild type plants, under conditions of limiting CO_2 saturation, such as low humidity and low CO_2 concentration (see Table 2, page 55). Further evidence of involvement of *ictB* gene in bicarbonate transport activity is provided by recent studies by myself, demonstrating enhancement of bicarbonate transport, resulting in increased

inorganic carbon fixation by transgenic tobacco plants expressing the *ictB* gene (see Figure 12, enclosed herein). Briefly, RubisCO activity was measured in wild type, and transgenic tobacco plants expressing the ictB gene, under conditions of low humidity (stomal closure, limited gas exchange), and thus, only partial activation of the enzyme complex by CO_2. RubisCO activity was expressed as rate of carboxylation, directly measuring nmol CO_2 fixed per nmol active sites. The transgenic plants (open circles) clearly had superior carboxylation rates under non-activated conditions (open circles) than the wild type controls (open squares). That this superior inorganic carbon fixation was due to an increased availability of CO_2 substrate, and not to alteration of Rubisco catalytic properties, is demonstrated by the kinetics (S/V vs. S) plots in the inset: note the higher reaction rate (V max) but similar substrate affinity (Km) of the Rubisco activity in transgenic and wild type plants. Thus, the expression of the *ictB* gene in the transgenic tobacco plants resulted in increased CO_2 availability under conditions of HCO_3^- transport dependent Ci acquisition. In a recent paper describing the work on the transgenic plants [Lieman-Hurwitz, J., Rachmilevitch, S., Mittler, R., Marcus Y., and A. Kaplan (2003) Enhanced photosynthesis and growth of transgenic plants that express *ictB*, a gene involved in HCO_3^- accumulation in cyanobacteria. Plant Biotechnology J. 1: 43-50] we provide yet further clear evidence for the higher CO_2 concentration at the site of Rubisco in the transgenic plants which express ictB. The CO_2 compensation point was lower in the transgenic *Arabidopsis* and tobacco plants. This can only be attained if the internal CO_2 concentration is higher in the plants expressing ictB.

Thus, it is my strong opinion that the ictB gene disclosed in the instant specification clearly encodes "a polypeptide having a bicarbonate transporter activity", as recited in now amended independent claims 1 and 16.

In the official action, the Examiner states that the specification fails to provide guidance for a nucleic acid that hybridizes to SEQ ID NO:2 and that encodes a protein with inorganic carbon fixation activity, methods of using it, and plants thereby obtained. The Examiner further states that identifying nucleic acids functionally related to a given nucleic acid is highly unpredictable, and that a great many proteins have "inorganic carbon fixation activity", requiring "undue trial and error experimentation of one of ordinary skill in the art".

The Examiner has also stated that the specification fails to provide adequate description of the claimed invention, since the "claims are broadly drawn to a multitude of DNA molecules that hybridize to SEQ ID NO:2, or that comprise "any variation of a portion of any size of SEQ ID NO:2...and the specification only describes a nucleic acid from *Synechococcus* that comprises SEQ ID NO:2".

I wish to point out that the restrictions imposed by now amended independent claims 1 and 16, namely, a "...polynucleotide encoding a polypeptide...having an amino acid sequence at least 95% homologous to the sequence as set forth in SEQ ID NO:3..." and a "polynucleotide encoding a polypeptide having a bicarbonate transporter activity..." constitute clear criteria by which candidate polynucleotides can be screened. Indeed, using the methodology described in the instant specification and in the Response to Official Action filed by the Applicant on November 29, 2002, for identifying sequences homologous to the ictB coding sequence (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:3), I have succeeded in identifying a number of highly conserved peptide domains (see hydropathy plots Fig 10a and 10b, enclosed herein) which are characteristic of the ictB protein and it's homologues from other species (see Fig 11, amino acid sequence alignment, enclosed herein).

Briefly, the IctB protein from *Synechococcus* PCC 7942 and homologous protein *Synwh0268* from *Synechococcus* sp Strain WH 8102 were analyzed for characteristic transmembrane (hydrophobic) and hydrophilic domains using the TopPred program. Identification of proteins having significant homology, and alignment of the amino acid sequences was performed using the CLUSTALW multiple alignment program.

Thus, I believe that we have demonstrated that, provided the teachings of the present invention, one of ordinary skill in the art would be expected to be able to make and use the nucleic acid constructs and selection methods disclosed therein without undue experimentation, and with a reasonable expectation of success.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false

statements may jeopardize the validity of the application or any patent issued thereon.

July 28, 2003

A. Kaplan

Prof. Aaron Kaplan

Encl.:

Professor Aaron Kaplan - Brief Curriculum Vitae

Born in Israel, Dec. 12, 1945. Married, three children.

1963-1966 Military service
1975 - Ph.D., The Hebrew University of Jerusalem (HUJ).
1976 -1977 Post Doctorate with Drs. O. Bjorkman and JA Berry, Carnegie Institute, Stanford University, USA
1977 - 1980 Lecturer, Department of Plant Sciences, HUJ.
1980 - 1984 Senior Lecturer, Department of Plant Sciences, HUJ.
1984 - 1989 Associate Professor, Department of Plant Sciences, HUJ.
1982 - 1984 Secretary, Botanical Society of Israel.
1985 - 1988 Head, Biological studies, HUJ.
1989 - Professor of Botany, HUJ.
1990 - 1994 Chairman, Department of Plant Sciences, HUJ.
1992 - 1998 Member International Committee for Photosynthesis.
1994- 1997 Scientific Director of the Botanical Garden, Mount Scopus, HUJ.
1996- 1998 Chairman, Environmental studies, HUJ.
2000 - Chairman, Department of Plant Sciences, HUJ.

Director of The Minerva Center for the study of arid Ecosystems
Member of The Avron-Evenari Minerva Center for Photosynthetic Research.

Publications 1999-

Kaplan, A., Reinhold, L. (1999) The CO_2-concentrating mechanism of photosynthetic microorganisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 539-570.

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Merquiol, E., Pnueli, L., Cohen, M., Simovitch, M., Goloubinoff, P., Kaplan, A. and R. Mittler (2002) Seasonal and diurnal variations in gene expression in the desert legume *Retama raetam*. Plant Cell Environ. 25:1627-1638.

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Vardi, A., Schatz, D., Beeri, K., Motro, U., Sukenik, A., Levine, A. and A. Kaplan (2002) Dinoflagellate-cyanobacterium communication may determine the composition of phytoplankton assemblage in a mesotrophic lake. Current Biology 12: 1767-1772.

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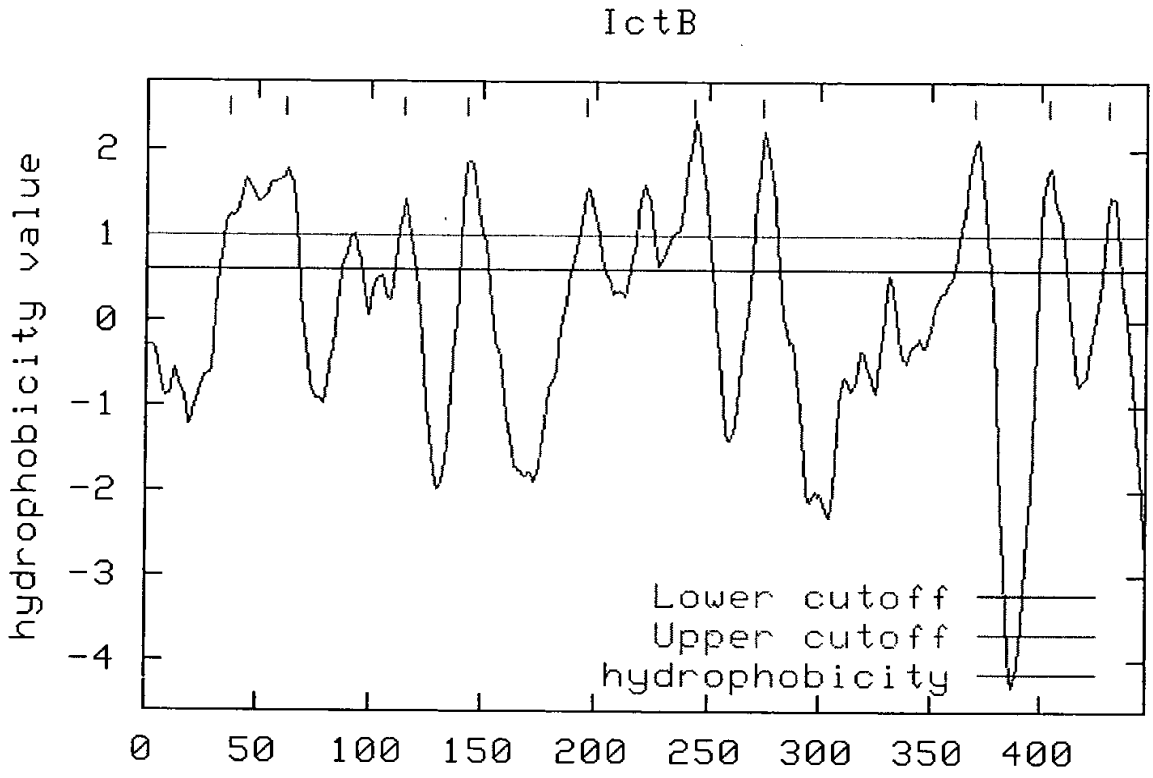
photoreduction of O_2 in cyanobacteria Current Biology 13: 230-235.

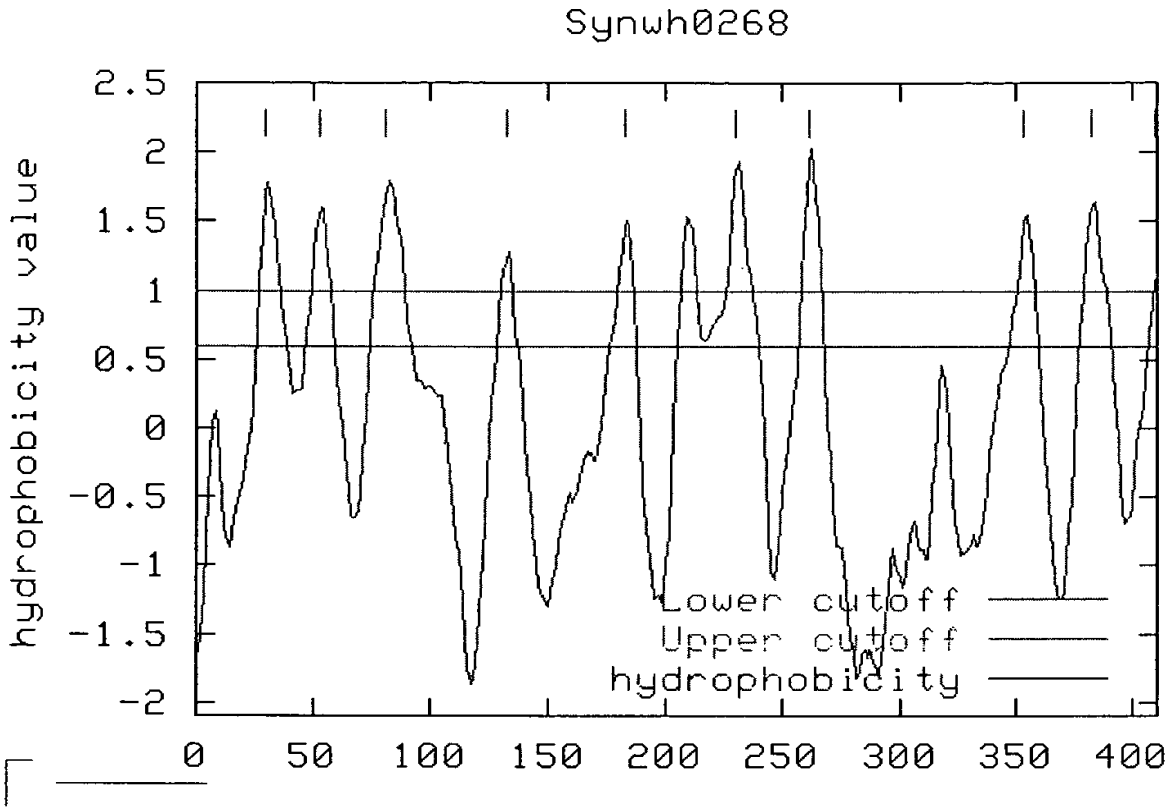
APPENDIX I

Figure 10: Hydropathy plots of two proteins, IctB from *Synechococcus* PCC 7942 and Synwh0268 from the marine *Synechococcus* sp. strain WH 8102 .

10a



Appendix II



10b

Appendix III

Figure 11: Alignment of ictB amino acid sequence with homologous cyanobacteria proteins.

Anabaena, gene product of all5073 from *Anabaena* sp. strain PCC7120 (SEQ ID NO:6);
Nostoc, Npun1329 from *Nostoc punctiforme* (SEQ ID NO:7);
Trichodesmium, a putative gene product from *Trichodesmium erythraeum* IMS101 (SEQ ID NO:10);
SLR1515, gene product of slr1515 from *Synechocystis* sp. strain PCC 6803 (SEQ ID NO:5);
IctB, gene product of *ictB* from *Synechococcus* sp. Strain PCC 7942 (the gene expressed in the transgenic plants) (SEQ ID NO:3)
Thermosyn, tlr2249 from *Thermosynechococcus elongatus* (SEQ ID NO:11);
Prochloroco., Pmit1577 from *Prochlorococcus marinus* strain MIT 9313 (SEQ ID NO: 12); and
Synechococcus, Synwh0268 from the marine *Synechococcus* sp. strain WH 8102 (SEQ ID NO:13).

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legend: * =Identity;: =strong similarity; . =similarity
10        20        30        40        50        60
                    |         |         |         |         |         |
Anabaena        ---MNLVWQRFTLSSLPLK--QFLATSYLHRFLVGLLSSWRQTSFLLQWGDMIAAALLSL
Nostoc          MFFMNLVWQLFTLSSLPLK--EYLATSYVHRSLVGLLSSWRQTSVLIQWGDAIAAVLLSS
Trichodesmium   ---MNSVWKKLTLTNLSFSDSEWLNASYLYGLLNGSLYNWRRGSWLMQWGEPLGFVLLAI
SLR1515         MVSPISIWRSLMFGGFSPQ--EWGRGSVLHR-LVGWGQSWIQASVLWPHFEALGTALVAI
IctB            ----MTVWQTLTFAHYQPQ--QWGHSSFLHR-LFGSLRAWRASSQLLVWSEALGGFLLAV
Thermosyn       --------MDVLLRRLDVEG--WRSHSGVGR-LLGLLQGWQEKSWLGRWLPSLAVLLVGL
Prochloroco     ----------MPKTAAPQ----------PLLLRWQGHIPSSEAMQMRLQWIAGLLLMM
Synechococcus   ----------MADATDQRS---------IPLLLRWQGCLTPTASVQQRLELLSGVVLML
                                            *         :      :.  ::

                        70        80        90        100       110       120
                         |         |         |         |         |         |
Anabaena        IYVLAPFVSSTLVGVLLIACVGFWLLLTLSDEPSSNNNSLVTPIHLLVLLYWGIAAVATA
Nostoc          IYALAPFASSTLVGLLLVACVGFWLLLTLSDEVTPANVSSVTPIHLLVLLYWGIAVIATA
Trichodesmium   VFTLAPFVNTTLIGFLLLASAGFWVLLKVSDN----TQEYLTPIHLLIFLYWSIATLAVV
SLR1515         IFIAAPFTSTTMLGIFMLLCGAFWALLTFADQ----PGKGLTPIHVLVFAYWCISAIAVG
IctB            VYGSAPFVPSSALGLGLAAIAAYWALLSLTDI----DLRQATPIHWLVLLYWGVDALATG
Thermosyn       VLVLAPLMPSGMIGMLLAAGSGFWLLWTLAGE----REGRWSGVHLLVLLYWGIALLATV
Prochloroco     LLATLPMLTRTGLGLTILAAGALWIIWGCVTP-----AGRIGSISSCLLVFFAIACLATG
Synechococcus   LLGSLPFVSRSGLGLELAAAGLLWLLWSLITP-----AKRLGAISRWVLLYLAIAWVCTG
                :    *:     :*. :      * :                  :   :: :  :  :..

                       130       140       150       160       170       180
                         |         |         |         |         |         |
Anabaena        LSPVKKAALTDLLTLTLYLLLFALCARVLR-SPRLRSWIITLYLSASLVVSIYGMRQWRF
Nostoc          LSPVKKAALNDLGTLTLYLLLFALCARVLR-SPRLRSWILTLYLHVSLIVSVYGLRQWFF
Trichodesmium   ISPAKTAAFSGWVKLTLYLLLFASGSLVLR-SPRLRSWLINIYLLVSLVVSFYGIRQWID
SLR1515         FSPVKMAAASGLAKLTANLCLFLLAARLLQ-NKQWLNRLVTVVLLVGLLVGSYGLRQQVD
IctB            LSPVRAAALVGLAKLTLYLLVFALAARVLR-NPRLRSLLFSVVVITSLFVSVYGLNQWIY
Thermosyn       LSPVPRAAMVGLGKLTLYLLFFALAERVMR-NERWRSRLLTVYLLTALMVSVEGVRQWIF
Prochloroco     FSPVPLAAAKGLIKLISYLGVYALMRQLLATSSDWWDRLVAALLTGELISSVIAIRQLYA
Synechococcus   FSPVPIAAAKGLLKLTSYLGVYALMRTLLERQIVWWDRLLAALLGGGLFSSVLALRQLYA
                :**.  **  .  .*   * .:     ::  .    . :.   :   *. .  .:.*

                                190       200       210       220       230
240
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                                 |         |         |         |         |
|
Anabaena      GAPPLATWVDPESTLSKTTRVYSYLGNPNLLAGYLVPAVIFSLMAVFVWQGWARKSLAVT
Nostoc        GATALATWVDPESPLSKTTRVYSYLGNPNLLAGYLLPAVIFSLVAIFAWQSWLKKALALT
Trichodesmium KVEPLATWNDPTSAQAGATRVYSYLGNPNLLGGYLLPAIALSFVAIFAWSSWARKSLAVT
SLR1515       GVEQLATWNDPTSTLAQATRVYSFLGNPNLLAAYLVPMTGLSLSALVVWRRWWPKLLGAT
IctB          GVEELATWVDRNSVADFTSRVYSYLGNPNLLAAYLVPTTAFSAAAIGVWRGWLPKLLAIA
Thermosyn     GAEPLATWTDPESALANVTRVYSFLGNPNLLAGYLLPSVPLSAAAIAVWQGWLPKLLAVV
Prochloroco   PAEEMAHWADPNSVAAGTVRIYGPLGNPNLLAGYLMPILPLALVALLRWQGLGAKLYAMV
Synechococcus STDELAGWADPNSVSAGTIRIYGPLGNPNLLAGYLLPLVPLACIAVLRWKRLSCRLLAAV
               .  :* * *  *    . *:*. *******..**:*   ::  *:  *     :  . .

                      250       260       270       280       290       300
                       |         |         |         |         |         |
Anabaena      MLFVNTACLIFTYSRGGWIGLVVAVLGATALLVDWWSVQMPPFWRTWSLPILLGGLIGVL
Nostoc        MLIVNTACLILTFSRGGWIGLVVAVLAVMALLVFWKSVEMPPFWRTWSLPIVLGGLIGIL
Trichodesmium ILLVSCACLRYTGSRGSWIGFLALMFAMLILMWYWWRSYMPSFWQIWSLPIAVGSFAGLL
SLR1515       MVIVNLLCLFFTQSRGGWLAVLALGATFLALCYFWWLPQLPKFWQRWSLPLAIAVAVILG
IctB          ATGASSLCLILTYSRGGWLGFVAMIFVWALLGLYWFQPRLPAPWRRWLFPVVLGGLVAVL
Thermosyn     MLGMNAASLILTFSRGGWLGLVAATIAGVVLLGIWFWPRLPLQWRRWGVPTMGGLAIALC
Prochloroco   ALGLGITATLFSFSRGGWLGMLSALAVILVLLLLRSTSHWPLVWRRLLPLIVIVLGTAML
Synechococcus TALLAGSATVFTYSRGGWLGLLAALALAGMLILLRTTAHWPPLWRRLLPLAALLIAGIAL
                     .   : ***.*:..:        *         *  *:

                      310       320       330       340       350       360
                       |         |         |         |         |         |
Anabaena      LIAVLFVEPVRFRVLSIFADRQDSSNNFRRNVWDAVFEMIRDRPIIGIGPGHNSFNKVYP
Nostoc        LLAVIFVEPVRLRVFSIFADRQDSSNNFRRNVWDAVFEMIRDRPIFGIGPGHNSFNKVYP
Trichodesmium ILAVVLLEPLRDRVLSVFAGRQDSSNNFRMNVWMSVFDMIRDRPILGIGPGNDVFNKIYP
SLR1515       GGALIAVEPIRLRAMSIFAGREDSSNNFRINVWEGVKAMIRARPIIGIGPGNEAFNQIYP
IctB          LVAVLGLEPLRVRVLSIFVGREDSSNNFRINVWLAVLQMIQDRPWLGIGPGNTAFNLVYP
Thermosyn     MGTIVSVPPLRERAASIFVARGDSSNNFRINVWMAVQQMIWARPWLGIGPGNVAFNQIYP
Prochloroco   VIAATQIEPIRTRITSLIAGRSDSSNNFRINVWLSSLEMIQARPWLGIGPGNAAFNRIYP
Synechococcus ALAITQLDPIRTRVLSLVAGRGDSSNNFRINVWLAAIEMVQDRPWLGIGPGNAAFNSIYP
                :   : *:* * *:..  * ******* *** .   *:  ** :*****:   **:**

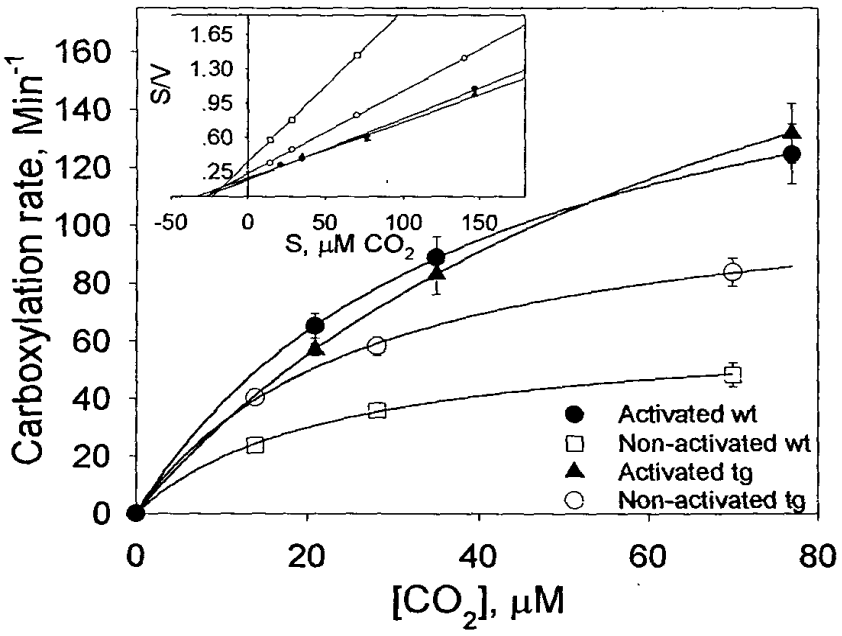
                      370       380       390       400       410       420
                       |         |         |         |         |         |
Anabaena      LYQR-PRYSALSAYSIFLEVAVEMGFVGLACFLWLIIVTINTAFVQLRQLRQSANVQGFW
Nostoc        LYQH-PRYTALSAYSILFEVTVETGFVGLACFLWLIIVTFNTALLQVRRLRRLRSVEGFW
Trichodesmium LYQR-PRYSALSSYSVPLEIVVETGFIGLTAFLWLLLVTFNQGVLQLKRLRDADNPQGYW
SLR1515       YYMR-PRFTALSAYSIYLEILVETGVVGFTCMLWLLAVTLGKGVELVKRCRQTLAPEGIW
IctB          LYQQ-ARFTALSAYSVPLEVAVEGGLLGLTAFAWLLLVTAVTAVRQVSRLRRDRNPQAFW
Thermosyn     LYQVNVRFTALGAYSIFLEILVEVGFIGFGVFLWLLAVLGDRARRCFEELRATGSPQGFW
Prochloroco   LFQQ-PKFNALSAYSVPLEILVETGLAGLMASLALVITGMRKGLAGLNSNHPL----ALP
Synechococcus LYQQ-PKFDALSAYSVPLEILVETGIPGLLACLGLLLSSIQRGLR-IHGQQ------GLI
               :    :: **.:**: :*: ** *. *:     *:      .   .   :      .

                      430       440       450       460       470       480
                       |         |         |         |         |         |
Anabaena      LVGALATLLGMLAHGTVDTIWFRPEVNTLWWLMVALIASYWTPLSANQCQELNLFKEEPT
Nostoc        LIGAIAILLGMLAHGTVDTVWYRPEVNTLWWLIVALIASYWTPLTQNQTNPS---NPEPA
Trichodesmium LIGAIAAMVGLIGHGLVDTVWYRPQVNTIWWLMVAIIASYSSQQGVRSRE----------
SLR1515       IMGALAAIIGLLVHGMVDTVWYRPPVSTLWWLLVAIVASQWASAQARLEASKEENEDKPL
IctB          LMASLAGLAGMLGHGLFDTVLYRPEASTLWWLCIGAIASFWQPQPSKQLPPEAEHSDEKM
Thermosyn     LMGTIAAMIGMLTHGLVDTIWFRPEVATLWWLMVAIVASFTPFQSKTANGTFSNRDPEP-
Prochloroco   ALASLAAIAGLAVHGITDTIFFRPEVQLVGWFCLATLAQTQPEQKQLQQTE---------
Synechococcus AIGSLAAIAGLLTQGITDTIFFRPEVQLIGWFALASLGATWLRD----------------
               :.::* : *:  :*  **: :** .  : *: :. :.
```

Anabaena	SN-
Nostoc	VN-
Trichodesmium	---
SLR1515	LAS
IctB	---
Thermosyn	---
Prochloroco	---
Synechococcus	---

Appendix IV

Figure 12: RubisCO activity *in vivo* (non-activated) and *in vitro* (activated) in wild type (wt) and transgenic (tg) tobacco plant in low humidity as a function of $[CO_2]$. Inset: kinetics (S/V vs. S) plots of RubisCO activity. n=6.



NCBI results of BLAST

BLASTN 2.2.6 [Apr-09-2003]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Sch?ffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1056873405-010515-31569

Query=
(946 letters)

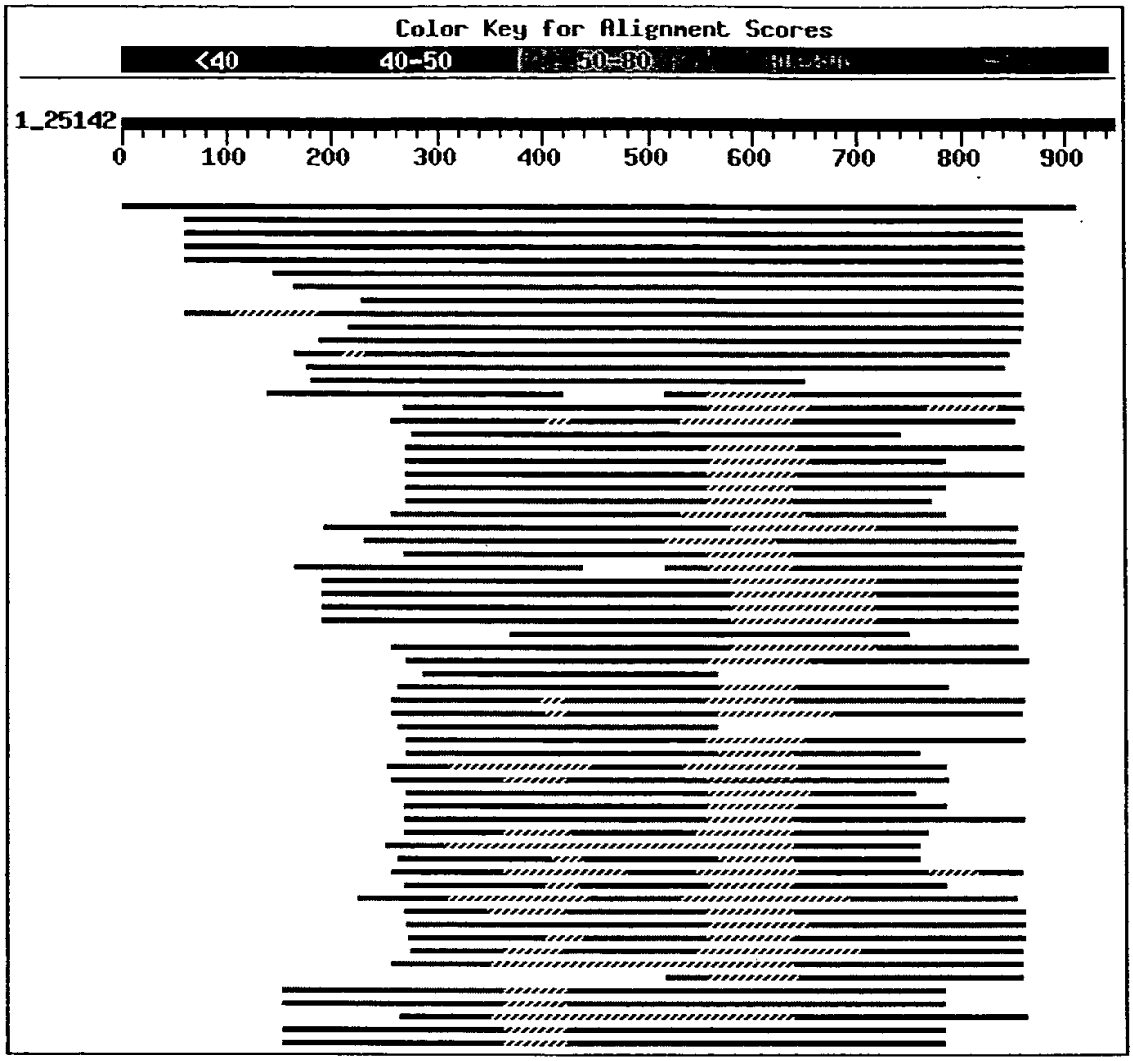
Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences)
1,810,334 sequences; 8,500,654,645 total letters

If you have any problems or questions with the results of this search please refer to the **BLAST FAQs**

Taxonomy reports

Distribution of 224 Blast Hits on the Query Sequence

Mouse-over to show defline and scores. Click to show alignments



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Alignments

Get selected sequences | Select all | Deselect all

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Strand = Plus / Plus

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Photosynthesis Research **00**: 1–11, 2003.

Inorganic Carbon Acquisition Systems in Cyanobacteria

Minireview

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Key words: CO_2-uptake, CO_2-concentrating mechanism, cyanobacteria, HCO_3^- transport, NADPH dehydrogenase

Abstract

This minireview focuses on the mechanism of inorganic carbon uptake in cyanobacteria and in particular the two CO_2 uptake systems and two bicarbonate transporters recently identified in *Synechocycstis* PCC 6803, and their presence in other cyanobacterial strains.

Introduction

Cyanobacteria possess a CO_2-concentrating mechanism (CCM) that enables efficient CO_2 fixation despite the low affinity of their Rubisco for CO_2 (Kaplan, Badger and Berry 1980; Ogawa, 1993; Kaplan and Reinhold 1999; Badger and Spalding 2000). The cellular components involved in the operation of the CCM include those engaged in inorganic carbon (Ci) uptake and accumulation, and the carboxysomes where most of the Rubisco and carbonic anhydrase (CA) are confined (Fukuzawa et al. 1992; Price, Colman and Badger 1992). It is well established that CO_2 and HCO_3^- are actively taken up by separate, independent systems (Volokita et al. 1984; Espie, Miller and Canvin 1989; Miller, Espie and Canvin 1990, 1991). Generation of CO_2 from the HCO_3^- accumulated within the cells is not catalyzed in the cytoplasm, and the Ci species do not reach equilibrium there (Reinhold, Zviman and Kaplan 1986; Price and Badger 1989). The accumulated HCO_3^- penetrates the carboxysomes where CA catalyzes the formation of CO_2 in close proximity to Rubisco. In addition to compensating for the relatively low affinity of Rubisco for CO_2, elevation of CO_2 concentration at the carboxylating site activates the enzyme and depresses photorespiration. The massive transmembrane Ci fluxes involved in the operation of the CCM could help dissipate excess light energy. High-CO_2-requiring mutants served as the main tool to identify genes involved in the operation of the CCM and to clarify many of the physiological processes. The first mutants isolated were impaired in various aspects related to the functional organization of the carboxysomes, these will not be discussed here (but see Marcus et al. 1986; Price et al. 1993; Ogawa, 1993, Kaplan and Reinhold 1999; Badger and Spalding 2000).

Recently, Ci-acquisition systems consisting of two CO_2-uptake mechanisms and two bicarbonate transporters were identified in *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis* 6803) and *Synechococcus* sp. strain PCC 7942 (hereafter *Synechococcus* 7942) and mutants impaired in these activities, or possessing only one Ci-acquisition system, became available (Omata et al. 1999; Ohkawa, Pakrasi, Ogawa 2000; Shibata et al. 2001, 2002a, 2002b; Maeda et al. 2002). Use of these mutants enabled detailed analysis of the physiological characteristics of each system. In this minireview we describe physiological characteristics and phylogenetic analysis of the four Ci acquisition systems identified in *Synechocystis* 6803. For a more comprehensive background, the reader is referred to earlier reviews (Miller, Espie and Can-

JS: DISK: CP: PIPS No. 5141143 (preskap:bio2fam) v.1.1
MS5.tex; 24/07/2003; 10:37; p.1

vin 1990; Ogawa, 1993; Raven 1997; Kaplan and Reinhold 1999; Badger and Spalding 2000).

Mutants defective in CO_2 uptake

It is widely accepted that light-dependent uptake of CO_2 in cyanobacteria involves conversion of CO_2 to HCO_3^- inside the cells (Volokita et al. 1984). It is assumed that this conversion is mediated by a component that possesses a carbonic anhydrase (CA)-like activity (Volokita et al. 1984; Kaplan and Reinhold 1999). Isolation of the high-CO_2-requiring mutants of *Synechocystis* 6803, RKa and RKb, defective in CO_2 uptake (Ogawa 1990) constituted the first breakthrough in the molecular analysis of CO_2-uptake systems in cyanobacteria. These mutants bear point mutations in *ndhB* and *ndhL* (renamed from *ictA*) encoding subunit proteins of NAD(P)H dehydrogenase (NDH-1) (Ogawa 1991a, 1991b, 1992). A Δ*ndhB* mutant of *Synechococcus* 7942 also showed a high CO_2-requiring phenotype (Marco et al. 1993). The observation that NDH-1 complexes involved in the conversion of CO_2 to HCO_3^- (see below) are localized on the thylakoid membrane (Ohkawa et al. 2001) suggested that CO_2 enters the cells by diffusion followed by conversion to HCO_3^- by a thylakoid-located mechanism. Mass-spectrometric measurements showed that the light-dependent ^{18}O exchange between CO_2 and water was strongly impaired in the RKa and RKb mutants (Ogawa 1990). Further, application of a water-channel blocker strongly inhibited CO_2 uptake in *Synechococcus* 7942 suggesting that CO_2 enters the cells by diffusion via water channels (Tchernov et al. 2001). Taken together, these data are consistent with the proposal that the maintenance of the concentration gradient driving the diffusive CO_2 influx depends on the conversion of CO_2 to HCO_3^- in the cytoplasm. Since CO_2 uptake shows characteristics of saturable kinetics, the Vmax is likely to be set by the maximal activity of the converting system.

Two functionally distinct NDH-1 complexes

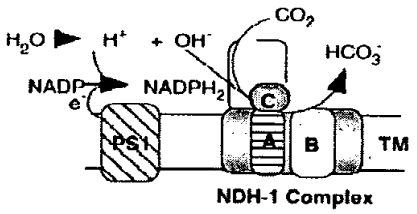
Analysis of the genomic sequence of *Synechocystis* 6803 revealed the presence of multiple copies of *ndhD* and *ndhF*, although most of the other *ndh* genes are present as a single copy (Cyanobase; http://www.kazusa.or.jp/cyano/). NdhD and NdhF are both members of a larger family and may be related to an ancient gene duplication event. Phylogenetic analysis indicated that at least four genes belong to the *ndhD* (*ndhD1, D2, D3* and *D4*) and three genes to the *ndhF* (*ndhF1, F3* and *F4*) families. Inactivation of each of the *ndhD* had little effect on the growth characteristics of the cells except that mutant Δ*ndhD3* grew very slowly under extremely low CO_2concentrations (i.e., 50 μL/L CO_2, Ohkawa et al. 1998; Price et al. 1998). Among the 4*ndhD* genes, *ndhD1* and *ndhD3* are highly homologous to *ndhD2* and *ndhD4*, respectively. The double mutant, Δ*ndhD1/ndhD2*, was unable to grow under photoheterotrophic conditions although it took up CO_2 in the light and grew normally under air levels of CO_2(Ohkawa, Pakrasi and Ogawa 2000). In contrast, the double mutant Δ*ndhD3/ndhD4* grew under photoheterotrophic conditions but was unable to take up CO_2 and to grow in air at pH 7.0 (Ohkawa, Pakrasi and Ogawa 2000). Thus, it became evident that *Synechocystis* 6803 possesses two functionally distinct NDH-1 complexes. The NDH-1 of the NdhD1/NdhD2 type mediates the electron transport from NADPH to plastoquinone and is an essential component of PS I-dependent cyclic electron flow. The contribution of NDH-1 type NdhD3/NdhD4 to this electron transport appears to be small. Table 1 summarizes the typical phenotypes of the Δ*ndhD1/ndhD2* and Δ*ndhD3/ndhD4* mutants. Notably, the Δ*ndhB* mutant (M55) exhibited all the phenotypes shown by the two double mutants suggesting that, functionally, NdhB is located upstream of the NdhD1/NdhD2 and NdhD3/NdhD4 systems (Ogawa 1991a).

Constitutive and low CO_2-induced CO_2 uptake systems

Northern analysis of the expression of *ndhD* genes revealed that *ndhD2* and *ndhD3* are induced by low-CO_2 whereas *ndhD1* and *ndhD4* are constitutively expressed (Ohkawa et al. 1998). These data raised the possibility that two CO_2 uptake systems operate in *Synechocystis* 6803, one NdhD3-dependent induced under low-CO_2 conditions and a second constitutive, NdhD4-dependent system. RT-PCR analyses indicated that in *Synechocystis* 6803 *sll1732 (ndhF3), sll1733 (ndhD3)* and *sll1734* (*cupA*, CO_2 *up*take A) are co-transcribed (Ohkawa et al. 2000). Mutants constructed by inactivation of each of these genes grew very slowly at 50 ppm CO_2 (Ohkawa et al. 1998) and mutants of *Synechococcus* PCC 7002 where *ndhD3, ndhF3*

Table 1. Phenotypes of mutants impaired in subunits of NADPH dehydrogenases

Phenotypes	M55 (ΔndhB)	ΔndhD1/ ndhD2	ΔndhD3/ ndhD4
Growth under photoheterotrophic conditions	no	no	yes
Respiration rate	low	low	high
Photosystem-1 cyclic electron transport	no	no	yes
Growth under low CO_2 at pH 7	no	yes	no
CO_2 uptake	no	yes	no



	Low CO_2 Induced	Constitutive
Components		
A	NdhD3	NdhD4
B	NdhF3	NdhF4
C	CupA	CupB
$K_{1/2}(CO_2)$(μM)		
H-cells	0.9	2.8
L-cells	0.9	2.8
Vmax (μmol/mg Chl h)		
H-cells	45	150
L-cells	320	135

Figure 1. A schematic model of the CO_2-uptake systems, their kinetic parameters and the components involved. The kinetic parameters were calculated from CO_2 uptake experiments performed with whole cells.

or *cupA* were inactivated showed a similar phenotype (Price et al. 1998). Regardless of the CO_2 concentration during growth, these mutants exhibited the CO_2-uptake characteristics of the constitutive system, i.e., relatively low affinity for CO_2 and low Vmax for CO_2 uptake (Klughammer et al. 1999; Ohkawa et al. 2000; Shibata et al. 2001, Figure 1). Transformation of a *ΔndhD3* mutant with a transposon-bearing library enabled the isolation of mutants unable to grow in air at pH 7.0. These mutants bore the antibiotic-resistance tags within *slr1302*, designated *cupB* (a homologue of *cupA*, Shibata et al. 2001). Mutants constructed by inactivation of *sll0026* (*ndhF4*), *sll0027* (*ndhD4*) or *cupB* (but possessing normal NdhD3-NdhF3-CupA) showed CO_2-uptake characteristics of the low-CO_2-induced system, i.e. higher affinity to CO_2 and Vmax typical of low CO_2-grown cells (Figure 1). Double mutants impaired in one component of each of these systems, the high- (induced) and the low affinity (constitutive), demanded high CO_2 for growth (Shibata et al. 2001, 2002a). Figure 1 shows a schematic model of the CO_2-uptake systems and the components involved. It also provides the kinetic parameters of the induced and the constitutive CO_2-uptake systems calculated from uptake experiments performed with whole cells. As expected, the apparent affinity of the low-CO_2 induced system for CO_2 ($K_{1/2}(CO_2)$ = 0.9 μM) was significantly higher than that exhibited by the constitutive one ($K_{1/2}(CO_2)$ = 2.8 μM). Notably, while the curves relating CO_2 uptake to its concentration showed saturable kinetics, a significant extended linear phase was detected (Shibata et al. 2001) suggesting that CO_2 uptake was diffusion-limited and that the intrinsic km(CO_2) of the uptake systems is even lower. The very high affinity for CO_2 could explain the observation that low-CO_2-grown cyanobacterial cells can utilize Ci from the medium almost to completion. It is also important to note (Figure 1) that the affinity of the low CO_2-induced system for CO_2 did not change during acclimation of the cells after transfer from high to low-CO_2. In contrast, the Vmax of uptake increased significantly during this acclimation. These data suggested that the acclimation to low-CO_2 involves a rise in the abundance of transporting entities rather than post-transcriptional modification of those already present under high CO_2 as earlier proposed for HCO_3^- transport (Sültemeyer et al. 1998).

An ABC-type HCO_3^- transporter (BCT1)

The finding of a low CO_2-inducible 42 kD protein in the cytoplasmic membrane of *Synechococcus* 7942 was the first step towards molecular analysis of

Table 2. Growth characteristics of *Synechocystis* 6803 mutants impaired in various Ci acquisition system(s)

Mutants	In air at pH 7.0	In air at pH 9.0
ΔA	+[a]	+
ΔB	+	+
ΔA/B	–	+
ΔA/B/C	–	+
ΔA/B/D	–	–
ΔA/B/C/D	–	–
ΔA/D	+[a]	+
ΔB/D	+	+
ΔC/D	+	+

A: Low CO_2-induced CO_2 uptake system; B: Constitutive CO_2 uptake system; C: ABC-type HCO_3^- transporter (BCT1); D: SbtA-type HCO_3^- transporter.
[a]Growth was very slow at 20 ppm CO_2.

HCO_3^- transport (Omata and Ogawa 1985, 1986). A gene encoding this protein (*cmpA*, *c*ytoplasmic *m*embrane *p*rotein) was isolated and shown to form an operon with *cmpB*, *cmpC* and *cmpD* (Omata et al. 1990; Omata 1992). Sequence data indicated that these genes encoded subunits of an ABC-type transporter, cmpA being a substrate-binding subunit. Experimental evidence that the *cmp* operon encodes an ABC-type HCO_3^- transporter was obtained after it was constitutively expressed in high CO_2-grown cells (Omata et al. 1999). The cmpA protein obtained by expressing the gene in *E. coli* possessed HCO_3^- binding capability (Maeda et al. 2000). The $K_{1/2}$ (HCO_3^-) value for the BCT1-dependent HCO_3^- transporter and the affinity of cmpA for HCO_3^-, estimated by a mass spectrometry, were 15 and 5 μM, respectively. Interestingly, inactivation of *cmp* genes in *Synechocystis* 6803, including strain *ΔndhD3/ndhD4*, hardly affected the HCO_3^- transport activity and the mutant showed the growth characteristics of the wild types at pH 9.0. (Shibata et al. 2001). These data suggested that the cmpA-D HCO_3^- transporter plays a minor role in *Synechocystis* 6803 and that another HCO_3^- transporting system operates in this organism.

A sodium-dependent HCO_3^- transporter

Cyanobacteria readily use CO_2 as a carbon source even at alkaline pH values, owing to their high affinity for CO_2 (particularly when grown under low CO_2 concentration). Therefore it has been difficult to detect and identify the HCO_3^- transporting entities. The use of mutants unable to take up CO_2 has proven helpful in this respect. The double mutant of *Synechocystis* 6803, *ΔndhD3/ndhD4*, was unable to take up $CO_2$and hence to grow at pH 7.0 in air. On the other hand, at pH 9.0 it exhibited normal HCO_3^--transport activity and could grow like the wild type in air (Ohkawa et al. 2000), conditions where Ci is mainly supplied by HCO_3^- transport. Sodium ions are essential for HCO_3^- uptake by cyanobacteria (Reinhold, Zviman and Kaplan 1986; Espie and Kandasamy 1994; So et al. 1998) and hence for their growth (Miller, Turpin and Canvin 1984) particularly at alkaline pH values. Recently, it was shown that *slr1512*, designated *sbtA* (*s*odium-*b*icarbonate *t*ransport), encodes a novel transporter involved in Na^+-dependent HCO_3^- uptake (Shibata et al. 2002b). Inactivation of *sbtA* in the wild type had no effect on the growth characteristics of cells grown under air in normal BG-11 medium. It is likely that under these conditions CO_2 uptake provided enough carbon to support growth. On the other hand, inactivation of *sbtA* in a *ΔndhD3/ndhD4* mutant (impaired in CO_2 acquisition) abolished Ci uptake almost completely and the cells were unable to grow in air. Disruption of *sbtA* in the single *ΔndhD3* or *ΔndhD4* mutants, which are able to take up CO_2 either by the constitutive or by the inducible systems, respectively (Shibata et al. 2001; Ohkawa et al. 2000a, b), hardly affected their growth. Table 2 shows that the presence of either low CO_2-inducible or constitutive CO_2-uptake systems is essential for growth at pH 7.0 in air. Most pronounced is the role of the low CO_2-inducible system for growth under very low CO_2 concentrations, lower than 1.5 μM CO_2. On the other hand, the SbtA-type HCO_3^- transporter was essential for growth at pH 9.0 in air when both CO_2-uptake systems were inactivated. The contribution of the ABC-type HCO_3^- transporter (BCT1) to *Synechocystis* 6803 growth is very small.

In high CO_2-grown *Synechocystis* 6803, the abundance of the transcript originating from *sbtA* was very low and in most cases hardly detectable (Shibata et al. 2002b). The level of this transcript increased significantly within 2 to 6 h of exposure to air level of CO_2 concomitant with a large rise in HCO_3^- transport activity. Maximal rate of SbtA-dependent HCO_3^- uptake was reached at 100 μM HCO_3^- and the $K_{1/2}$(HCO_3^-) value was about 16 μM (Figure 2). SbtA-mediated HCO_3^- transport was specifically dependent on the presence of Na^+ ions, maximal HCO_3^- uptake was attained at 6 mm $Na^+$and the concentration of Na^+ essential to support half maximal

HCO_3^- transport was about 1 mm. Three different alternatives were proposed to explain the specific dependence of HCO_3^- transport on the presence of sodium ions in cyanobacteria (Kaplan et al. 1990; Espie and Kandasamy 1994). That Na^+ is essential for the maintenance of the internal pH, via a Na^+/H^+ antiporter, during CO_2 fixation from imported HCO_3^-; that HCO_3^- is transported via a Na^+/HCO_3^- symporter secondary to a primary Na^+ pump that maintains the $\Delta\mu Na^+$ essential for fueling HCO_3^- transport; and that Na^+ binds to the HCO_3^- transporter and changes its affinity. The SbtA-dependent HCO_3^- uptake was strongly affected by the ambient pH, it was highest at pH 9 and the activity declined to about 50% and 20% at pH 8.0 and 7.0, respectively. This finding seems to exclude the possibility that the role of Na^+ is in the maintenance of the internal pH. Inactivation of *slr1509* (*ntpJ*, encoding a protein homologous to a subunit of HKT1 in *ArabidoPS Is thaliana* that mediates Na^+ transport, Uozumi et al. 2000) in the wild type abolished its ability to grow at Na^+ concentration higher than 0.1 m; and that of mutant *ΔndhD3/ndhD4* to grow in air but not its ability to grow under 3% CO_2. The HCO_3^--transport activity in the *ΔndhD3/ndhD4/ntpJ* mutant was only about one third that in the *ΔndhD3/ndhD4* mutant (Shibata et al. 2002b), supporting the notion that NtpJ is a subunit of a Na^+-extrusion pump essential for the SbtA-mediated HCO_3^- transport. The specific dependence of the SbtA-mediated HCO_3^- transport on $[Na^+]$ and the impaired ability of *ΔntpJ* mutant to transport HCO_3^- point to $\Delta\mu Na^+$ across the cytoplasmic membrane as the driving force for the SbtA-mediated HCO_3^- transport and suggest, therefore that SbtA functions as a Na^+/HCO_3^- transporter.

It was earlier proposed that IctB is involved in HCO_3^- uptake in *Synechococcus* 7942 suggesting that its homologue, Slr1515, may have a similar function in *Synechocystis* 6803 (Bonfil et al. 1998). However, lack of HCO_3^- uptake in the *ΔndhD3/ndhD4/sbtA/cmpA* mutant (Shibata et al. 2002b) did not lend support to this possibility. On the basis of results obtained by mass-spectrometry, it was concluded that *Synechococcus* sp. strain 7002 and *Synechococcus* 7942 possess a constitutive, low affinity, HCO_3^- transport system (Sültemeyer et al. 1998; Price et al. 2002). Interestingly, none of the HCO_3^- transporters recognized in *Synechocystis* 6803 (using mutants impaired in CO_2 uptake) is constitutively expressed. Although we once observed low levels expression of *sbtA* in high CO_2-grown cells, repeated experiments indicated that the transcript was hardly detectable in most cases. High CO_2-grown cells of*Synechocystis* 6803 did not exhibit low affinity HCO_3^- transport. This discrepancy might be due to differences between the various strains examined or the intrinsic difficulties involved in the assessment of HCO_3^- uptake by the mass spectrometry (see below).

Photosynthetic electron transport involved in CO_2 uptake and HCO_3^- transport

Clearly, the active uptake and accumulation of Ci to values 1000-fold higher in the cells than in their medium, is driven by photosynthetic light energy. However, the specific role of the photosynthetic reaction centers and of various segments of the photosynthetic electron transport chain is not understood. Action spectra for Ci accumulation indicated that uptake can be energized by Photosystem I (PS I) alone. Mutants of *Synechocystis* 6803 (Ogawa 1991a, b; Mi et al. 1992, 1995) and *Synechococcus* 7942 (Marco et al. 1993) defective in *ndhB* and *ndhL* (encoding subunits of NAD(P)H dehydrogenase, NDH-1) do not exhibit either PS I-cyclic electron transport or CO_2 uptake. Results obtained with these mutants supported the notion that NDH-1 dependent cyclic electron transport is essential to energize CO_2 uptake (Ogawa 1993). However, the finding that cyanobacteria possess two functionally distinct NDH-1 complexes and that inactivation of NdhD1/NdhD2-type NDH-1 complex or NdhF(1)-type NDH-1 complex essential to cyclic electron transport had little effect on CO_2 uptake (Sültemeyer et al. 1997; Ohkawa, Pakrasi and Ogawa 2000) made the direct involvement of cyclic PS I in CO_2 uptake questionable.

Use of specific electron transport inhibitors and acceptors suggested that CO_2 uptake in *Synechococcus* UTEX 625 is in fact supported by cyclic electron flow and that, in contrast, HCO_3^- transport depends on linear electron flow (Li and Canvin 1998). Draining of electrons from PS I to N, N-dimethyl-p-nitrosoaniline (PNDA) or methylviologen inhibited CO_2 uptake but not HCO_3^- transport (Ogawa, Miyano and Inoue 1985; Li and Canvin 1998; Tchernov et al. 2001). In both *Synechocystis* 6803 and *Synechococcus* 7942, CO_2 uptake by the low-CO_2-inducible system was severely inhibited by DCMU but the constitutive system was less sensitive (Shibata et al. 2002a, Maeda et al. 2002). Inhibition of CO_2 uptake by DCMU was alleviated by duroquinone (Tchernov

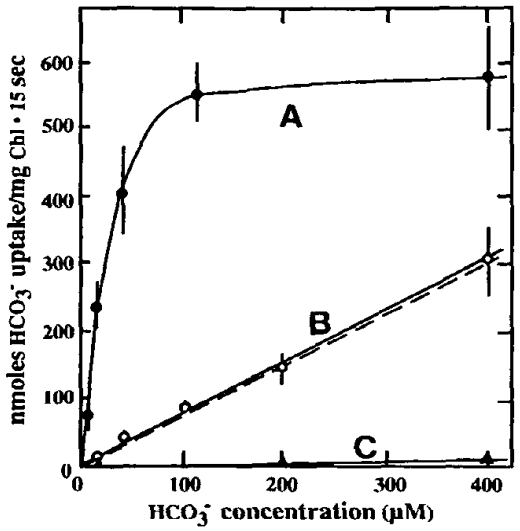


Figure 2. Uptake of HCO_3^- by the *ΔndhD3/ndhD4/cmpA* (A), *ΔcmpA/sbtA* (B) and *ΔndhD3/ndhD4/cmpA/sbtA* (C) strains as affected by the HCO_3^- concentration. Uptake was measured in the medium containing 15 mm NaCl and various concentrations of HCO_3^- at pH 9.0. Vertical bars indicate standard deviations (n = 5). Broken line shows the amount of CO_2 that could be produced spontaneously from HCO_3^- at pH 9.0.

et al. 2001) and iodoacetamide, which blocked CO_2 fixation and lowered the intracellular NADP/NADPH ratio (Ogawa, Miyano and Inoue 1985). It is possible that the low-CO_2-inducible system is supported by PS I activity that depends on electrons donation from PS II (such as reduction of NADP) whereas the constitutive system is partially supported by cyclic PS I electron transport. The data at hand are all consistent with the notion that reduction of NADP to NADPH is essential for CO_2 uptake, particularly that driven by the low-CO_2-induced system. Since it has been proposed that conversion of CO_2 to HCO_3^- depends on local alkalization or removal of protons (Kaplan and Reinhold 1999; Maeda et al. 2002), ferredoxin-NADP reductase, thought to be involved in cyclic electron transfer under high energy demanding conditions (Matthijs et al. 2002), might play a role.

Light-driven net CO_2 efflux during photosynthesis was observed in the marine *Synechococcus* strain WH 7803 consequent on dehydration of HCO_3^- within the cells (Tchernov et al. 1997). CO_2 efflux was also observed in the light in *Synechococcus* strains UTEX 625 and PCC 7942 following inhibition of CO_2 uptake by PNDA or methylviologen (Li and Canvin 1998; Tchernov et al. 2001). In contrast, in mutants capable of HCO_3^- transport but impaired in both the low CO_2-induced and the constitutive CO_2-uptake systems, efflux of CO_2 due to dehydration of accumulated HCO_3^- within the cells was largely reduced. Taken together, the data suggested that the thylakoid-located conversion systems contribute importantly to the formation of CO_2 from HCO_3^- within the cells (in addition to the carboxysomal-located CA) and that the direction of this CA-like activity (hydration or dehydration) is determined by the energization and redox states of the cells. The data also pointed to a massive Ci cycling between the cells and their medium driven by CO_2- and HCO_3^- -uptake systems (see Tchernov et al. this issue)

Both cyclic and linear electron transport lead to the formation of $\Delta\mu H^+$ across the thylakoid membrane, essential for the formation of ATP. The latter can energize the ABC-type HCO_3^- transporter and may also be used to produce the $\Delta\mu Na^+$ required to fuel the SbtA-dependent HCO_3^- transport. The mechanism by which CO_2 uptake is energized is not clear but recent studies indicated that $\Delta\mu H^+$ rather than ATP serves as the direct source of energy for the conversion of CO_2 to HCO_3^- (Tchernov et al. 2001). Conversion of CO_2 to HCO_3^- in alkaline domains could be used to energize CO_2 uptake (Kaplan and Reinhold 1999). Formation of these domains is possibly coupled to reduction of NADP to NADPH on the thylakoid, or also envisaged as extraction of protons at CA sites on this membrane (Maeda et al. 2002).

Suitability of the methods used for measuring CO_2 and HCO_3^- uptake

Isolation and characterization of mutants defective in CO_2 or HCO_3^- uptake or both enable us to revisit some of the problems encountered in the application of the various methods used to measure these fluxes. The silicon-oil filtering centrifugation (Volokita et al. 1984) and the filtering method (Omata et al. 1999) were used to measure total Ci uptake. These methods can also be used to measure the initial rates of CO_2 and of HCO_3^- uptake under isotopic disequilibrium conditions where labeled Ci is provided primarily as $^{14}CO_2$ or $H^{14}CO_3^-$ (Volokita et al. 1984; Miller, Espie and Canvin 1990). The filtering centrifugation method also enables the assessment of the kinetic parameters for the unidirectional influx of CO_2 and of HCO_3^-. Use of the open gas-exchange method

(Ogawa, Miyano and Inoue 1985) allows accurate measurement of the concentration of CO_2 leaving the cell suspension and hence calculation of the net rate of CO_2 uptake since, at steady state, the concentration of CO_2 in the medium remains constant. Use of this method showed that mutants capable of HCO_3^- uptake but unable to take up CO_2 do not exhibit net CO_2 uptake from the medium during photosynthesis (Ohkawa et al. 2000; Shibata et al. 2001). The mass-spectrometry method detects the change of CO_2 concentration in the closed chamber with time of consumption or evolution (Badger, Palmqvist and Yu 1994). This method was also used to calculate the net contribution of HCO_3^- uptake to the photosynthetic activity. However, use of this approach to assess the actual amount of HCO_3^- transported and/or its kinetic parameters may lead to significant underestimations of the Vmax and $K_{1/2}(HCO_3^-)$ due to the fact that HCO_3^- efflux is substantial (Kaplan and Reinhold 1999; Tchernov et al. 1997; Tchernov et al. this issue) and not negligible as originally proposed.

Figure 2 shows the rate of Ci uptake by three types of *Synechocystis* 6803mutants as affected by the HCO_3^-concentration, measured at pH 9.0 using the filtering method. There was no uptake of Ci in mutant Δ*ndhD3/ndhD4/cmpA/sbtA* (where the CO_2 and HCO_3^- uptake systems described above were inactivated) even at 400 μM HCO_3^-. These data suggested that *Synechocystis* 6803 does not possess an additional, possibly low affinity, HCO_3^- transporter. Mutant Δ*ndhD3/ndhD4/cmpA* where only the SbtA-dependent HCO_3^-transport is functional showed saturable kinetics and reached maximum uptake activity at approximately 100 μM HCO_3^-. In contrast, CO_2 uptake at pH 9.0 by mutant Δ*cmpA/sbtA* (unable to take up HCO_3^- but possessing functional CO_2-uptake systems) increased linearly with the ambient Ci concentration (but note that the CO_2 concentrations applied here were below the $K_{1/2}$ for CO_2 uptake; Shibata et al. 2001).The rate of CO_2 uptake was similar to the maximal rate of uncatalyzed CO_2 formation at this pH (Figure 2), indicating that uptake was rate-limited by the physicochemical conversion of HCO_3^- to CO_2 at the cell surface. In cyanobacteria, formation of CO_2 from HCO_3^- in close vicinity to the cytoplasmic membrane might be catalyzed by a periplasmic-located carbonic anhydrase (Bedu, Beuf and Jose 1992) or accelerated by light-dependent proton extrusion that could acidify the periplasmic space (Kaplan, Lerner and Scherer 1989; Scherer, Hinrichs and Böger 1988; Katoh et al. 1996). If the cells take up the CO_2 as it is formed in the periplasmic space, the amount diffusing outwards to the bulk medium (where it can be detected by the mass spectrometer) will be greatly reduced. If this CO_2 taken up is fixed in photosynthesis, it would erroneously be accounted for as HCO_3^- uptake.

Phylogeny of the Ci acquisition systems in cyanobacteria

The presence or absence of genes homologous to *ndhD3*, *ndhD4*,*ndhF3*, *ndhF4*, *cupA* and *cupB*, *sbtA* and*cmp* may provide better understanding of Ci uptake mechanisms in phytoplankton species and of their phylogenetic relationship. Inducible and constitutive CO_2 uptake-systems appear to be present in a number of cyanobacteria. Involvement of NdhD3 and NdhF3 in high affinity CO_2 uptake in *Synechococcus* sp. PCC 7002 has been reported (Klughammer et al. 1999). Mutants of *Synechococcus* 7942 impaired in *cupA* and *cupB* (in this organism designated *chpY* and *chpX*, respectively) also showed CO_2 uptake characteristics of the constitutive and low CO_2-induced systems, respectively (Maeda et al. 2002; Price et al. 2002). The exact role of CupA and CupB is not known. In mutants of *Synechococcus* 7942 where both *cupA* and *cupB* were inactivated, inter-conversion between CO_2 and HCO_3^- was slower than in the wild type. However, this does not necessarily indicate that the proteins encoded by these genes have a CA activity. In mutants RKa and RKb of *Synechocystis* 6803 likely to possess functional Cup proteins, interconversion between CO_2 and HCO_3^- was strongly inhibited (Ogawa 1990). Moreover, we were unable to detect any CA activity with soluble CupA protein expressed in *E. coli* (unpublished). Thus, the naming of *chpX* and *chpY* (*C*O_2 *h*ydration *p*rotein), based on the mass-spectrometric measurement of the mutant cells, is not justified. The phylogenetic analysis of NdhD/NdhF suggested an evolutionary relationship between cyanobacterial *ndhD1/ndhD2*-type and *ndhD* genes in chloroplast genomes; also that the cyanobacterial *ndhF1* is related to the chloroplast *ndhF* (Shibata et al. 2001). The analysis indicated that the *ndhD3*- and*ndhD4*-types, the *ndhF3/ndhF4*-type and the *cupA/cupB*-type genes specifically engaged in CO_2 uptake are present only in cyanobacteria. All the cyanobacterial species for which the whole genome sequence is available, with the exception of the marine *Synechococcus* and *Prochlorococcus marinus*, possess

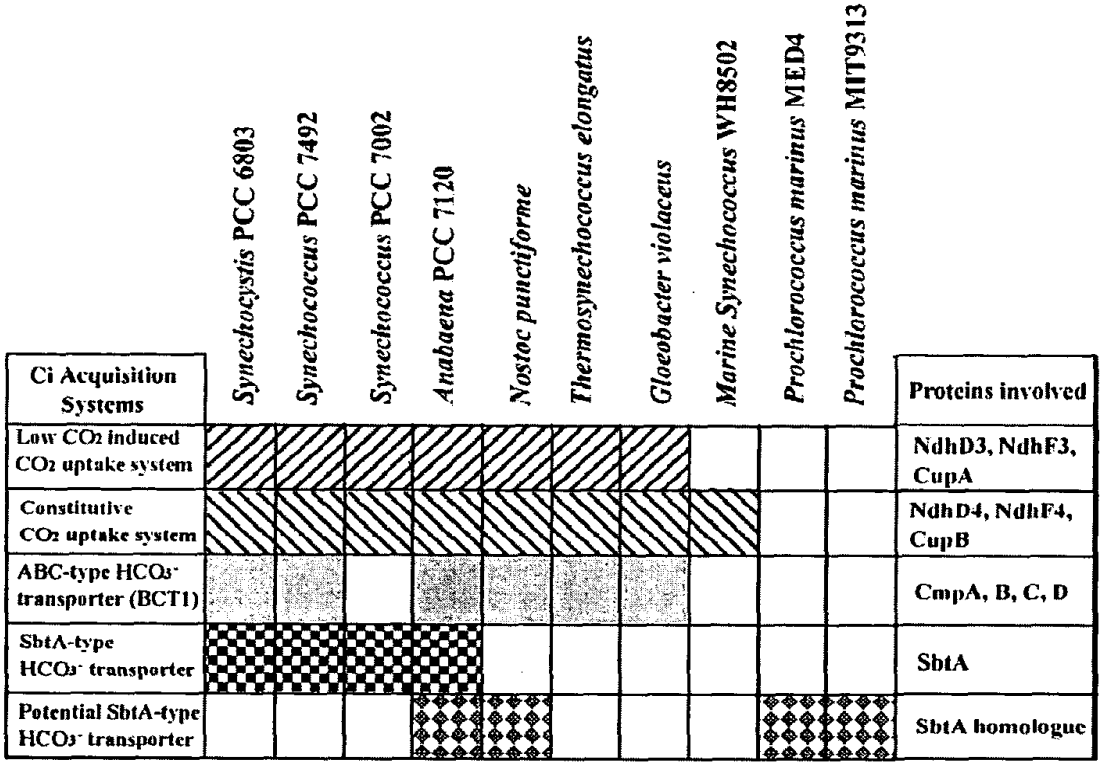


Figure 3. Presence of Ci acquisition systems in various cyanobacterial strains. Shadowed and open boxes indicate the presence and absence of each system, respectively.

both *ndhF3* and *ndhF4* genes and the *cupA*- and *cupB*-type genes (Figure 3). The marine *Synechococcus* does not possess homologues of *ndhD3/ndhF3/cupA* genes and, therefore, appears to be deprived of the low-CO_2-inducible CO_2-uptake system. The phylogenetic analysis also suggested that apart from passive uptake of CO_2 the low- and the high-light strains of *P. marinus*, MED4 and MIT9313, must relay on HCO_3^- transport as the sole source of Ci since they lack the *ndh* genes involved in active CO_2 uptake typical of other cyanobacteria (and possess only the *ndhD1*-type essential for PS I cyclic electron transport).

The ABC-type HCO_3^- transporter (BCT1) encoded by the *cmp* operon is present in *Synechococcus* 7942, *Synechocystis* 6803, *Anabaena* PCC 7120, *Nostoc punctiforme, Thermosynechococcus elongatus and Gloeobacter violaceus* but is absent in the marine cyanobacterial strains (*P. marinus* strains MED4 and MIT9313 and *Synechococcus* sp. PCC 7002) (Omata et al. 2002). It is intriguing to speculate that marine organisms or halotolerants like *Synechocystis* 6803 that can rely on a primary Na^+ pump to establish a $\Delta\mu Na^+$ as the driving force to fuel a Na^+/HCO_3^- transporter whereas fresh water strains use ATP to fuel an ABC type pump to drive HCO_3^- transport. Homologues of SbtA have been identified in *Synechococcus* sp. PCC 6301, *Synechococcus* sp. PCC 7002, *Anabaena* PCC 7120, *Nostoc punctiforme, P. marinus* strains MED4 and MIT9313 (Shibata et al. 2002b). There are two types of SbtA in cyanobacteria, one consisting of 370-374 and the other of 324-339 amino acids. *Anabaena* possesses both types of SbtA. The sequence homology between the 2 types of SbtA is relatively weak but analyses of hydrophobicity profiles indicated that both types contains 10 membrane-spanning domains that are structurally highly conserved. Whether the short

type SbtA is functioning as a HCO_3^- transporter is yet to be examined. As indicated, a SbtA-like HCO_3^- transporter was found in *P. marinus* strains; this important organism does not appear to possess any other Ci acquisition systems. Eukaryotic algae including *Chlamydomonas reinhardtii* possess light-dependent Ci uptake machinery (Kaplan and Reinhold, 1999; Badger and Spalding 2000). However, they probably depend on CO_2-uptake systems different from those functioning in cyanobacteria since homologues of the cyanobacterial *cup* genes were not detected there.

Concluding remarks

The cyanobacterial CCM comprises processes which accumulate Ci within the cells and raise the concentration of CO_2 at carboxylation sites within the carboxysomes. The past five years has seen significant advances in the recognition at molecular level of the systems engaged in HCO_3^- transport and CO_2 uptake and in our understanding of their dependence on light energy. On the other hand, little progress has been made towards better understanding of the assemblage of the carboxysomes, although the first, and for many years the only, cyanobacterial high-CO_2-requiring mutants were impaired in the structural organization of these bodies. Further, information is still missing on the nature of the CO_2 sensor and the processes involved in the acclimation of cyanobacteria to changing ambient CO_2 concentration. Recent identification of a transcription factor which plays a key role in the signal transduction pathway involved in the acclimation of *Chlamydomonas reinhardtii* to low CO_2 (Xiang, Zhang and Weeks 2001; Fukuzawa et al. 2001) may open the way to identify similar proteins in cyanobacteria.

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Genes Essential to Sodium-Dependent Bicarbonate Transport in Cyanobacteria: Function and Phylogenetic analysis*

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SUMMARY

The cyanobacterium *Synechocystis* sp. strain PCC 6803 possesses two CO_2-uptake systems and two HCO_3^- transporters. We transformed a mutant impaired in CO_2 uptake and in *cmpA-D* encoding a HCO_3^- transporter with a transposon-inactivation library, and recovered mutants unable to take up HCO_3^- and grow in low CO_2 at pH 9.0. They are all tagged within *slr1512* (designated *sbtA*). We show that SbtA-mediated transport is induced by low CO_2, requires Na^+, and plays the major role in HCO_3^- uptake in *Synechocystis*. Inactivation of *slr1509* (homologous to *ntpJ* encoding a Na^+/K^+ translocating protein) abolished the ability of cells to grow at [Na^+] higher than 100 mM and severely depressed the activity of the SbtA-mediated HCO_3^- transport. We propose that the SbtA-mediated HCO_3^- transport is driven by $\Delta\mu Na^+$ across the plasma membrane, which is disrupted by inactivating *ntpJ*. Phylogenetic analyses indicated that two types of *sbtA* exist in various cyanobacterial strains, all of which possess *ntpJ*. The *sbtA* gene is the first one identified as essential to Na^+-dependent HCO_3^- transport in photosynthetic organisms and may play a crucial role in carbon acquisition when CO_2 supply is limited, or in *Prochlorococcus* strains that do not possess CO_2-uptake systems or Cmp-dependent HCO_3^- transport.

INTRODUCTION

Growth of many photosynthetic microorganisms depends on the activity of a CO_2-concentrating mechanism (CCM)[1] which raises the $[CO_2]$ in close proximity to ribulose-1,5-bisphosphate carboxylase/oxygenase and thereby enables efficient CO_2 fixation despite the low affinity of the enzyme for CO_2 (1, 2). In the cyanobacterium *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis* 6803), the CCM involves active CO_2 uptake and HCO_3^- transport. We have recently identified two systems for CO_2 uptake in *Synechocystis* 6803, one constitutive and the other inducible by low CO_2 (3). As deduced from phylogenetic analysis of proteins encoded by the genes involved, these CO_2-uptake systems are present in various cyanobacteria with the exception of *Prochlorococcus marinus* (3). The inducible system that depends on NdhD3/NdhF3/CupA shows higher maximal activity and higher affinity for CO_2 than the constitutive, NdhD4/NdhF4/CupB-dependent system. Inactivation of two different genes, one encoding a component of the constitutive system, and the other a constituent of the inducible system, abolished CO_2-uptake activity. The double mutants were unable to grow at pH 7.0 under air level of CO_2 (3, 4). In contrast, since the mutants possessed HCO_3^- transport capability they could grow like the wild-type (WT) at pH 9.0 in air.

An ABC-type HCO_3^- transporter encoded by *cmpABCD* has been identified in *Synechococcus* sp. strain PCC 7942 (thereafter *Synechococcus* 7942) (5). Inactivation of *cmp* genes in *Synechocystis* 6803, however, had little effect on the HCO_3^- transport activity. This indicated that another HCO_3^- transporter, yet unidentified, plays a central role in HCO_3^- uptake. Sodium ions are essential for cyanobacterial growth, particularly at alkaline pH values (6), and they were implicated in HCO_3^- uptake (7). These results are consistent with the suggestion that

a Na^+-dependent HCO_3^- transporter might be functioning in cyanobacteria (7-10). In this paper we bring evidence that *slr1512* (designated *sbtA* for sodium-bicarbonate transport A) and *slr1509* (*ntpJ*) are essential for Na^+-dependent HCO_3^- transport and that *sbtA* most likely encodes a novel HCO_3^- transporter, the first one identified in photosynthetic organisms. We suggest that SbtA-mediated HCO_3^- transport could be driven by the electrochemical gradient of Na^+ across the plasma membrane, established by NtpJ.

EXPERIMENTAL PROCEDURES

Growth Conditions—WT and mutant cells of *Synechocystis* 6803 were grown at 30°C in BG11 medium (11) containing 20 mM N-cyclohexyl-2-aminoethanesulfonic acid (CHES)-KOH, pH 9.0, and bubbled with either 3% CO_2 in air (v/v) or air alone. Solid medium contained BG11 buffered at pH 9.0 and was supplemented with 1.5% agar and 5 mM sodium thiosulfate. Continuous illumination was provided by fluorescent lamps (50 μmol photons m^{-2} s^{-1}; ~~400-700 nm~~).

Construction and Isolation of Mutants—B1 is the mutant where several nucleotides within *ndhB* were replaced, as previously described (12,13). This mutant does not take up CO_2 but showed normal HCO_3^--transport activity. Construction of mutants *ΔndhD3*, *ΔndhD4*, *ΔcmpA* and *ΔntpJ* has been described earlier (12) and/or deposited in the web site "CyanoMutants" (http://www.kazusa.or.jp/cyano/mutants/). Strains bearing multiple mutations were obtained following transformation of given *Synechocystis* 6803 mutants with the constructs used to generate other single mutants.

A Genomic Priming System (New England Biolbs) was used to mobilize a transposon containing chloramphenicol-resistance (Cm^R) gene for random insertion into the DNA of 110 different cosminds, which contained DNA fragments of *Synechocystis* 6803 previously used for genomic sequencing (14). The B1/*ΔcmpA* mutant, defective in active CO_2 uptake, was transformed with this transposon inactivation library. Colonies formed on plates containing chloramphenicol, kanamycin and spectinomycin were transferred to duplicate plates buffered at pH 9.0 containing the same drugs. One plate was placed under 3% CO_2 in air (v/v) and the other in air alone. Mutants growing under 3% CO_2, but not in air, were recovered. The exact position of the Cm^R cassette in the mutant genome was determined as described previously (3).

Measurements of HCO_3^- Uptake and O_2 Evolution—The rate of HCO_3^- uptake was measured using $H^{14}CO_3^-$ in an assay buffer (50 mM CHES-KOH for pH 9.0 or N-Tris[hydroxymethyl] methyl-2-amino-ethanesulfonic acid-KOH for pH 7.0 and 8.0 containing 15 mM NaCl, 0.3 mM $MgSO_4$, 0.26 mM $CaCl_2$ and 0.22 mM K_2HPO_4) as previously reported (5). HCO_3^- uptake was initiated by the addition of $NaH^{14}CO_3/KHCO_3$. The sample was immediately illuminated with white light (400 μmol photons $m^{-2}s^{-1}$). Uptake was terminated by rapid filtration of the cells onto a glass filter (GF/B, Whatman) by suction, followed by immediate washing of the filter with 5 ml of the assay buffer. Oxygen evolution was measured with an O_2 electrode (Rank Brothers, Cambridge, England) on cells suspended in BG11 medium (pH 9.0) containing 15 mM NaCl. Cell suspensions (corresponding to Chl concentration of 10 μg/ml) were illuminated with white light (400 μmol photons $m^{-2}s^{-1}$) and, when O_2 evolution ceased, $NaHCO_3^-$ was added stepwise to attain the final concentrations of 5, 15, 30, 100 and 400 μM,

respectively.

RT-PCR Analysis of Expression—The amount of transcripts was evaluated by the RT-PCR method (15). RNAs were extracted from *Synechocystis* 6803 cells grown under 3 % CO_2 or after 2 and 6 hrs of bubbling with air, by the method of Aiba et al. (16), treated with RNase-free DNase I (Boehringer Mannheim) and then purified by phenol/chloroform extraction and ethanol precipitation. Reverse-transcription reaction was performed using superscript II (Gibco BRL) and reverse primers. The products were amplified by PCR and then analyzed by electrophoresis on 0.8 % agarose gel. Primers were designed so that the amplified products would be internal to the coding region of the genes. All the forward primers were designed for the sequences downstream of the translation initiation codon and the reverse primers to obtain the PCR products of about 350 bp. RNaseP gene was used as a control template (17). Reverse transcriptase was omitted from the RT reaction mixture to confirm the absence of contamination of genomic DNA.

Other Methods—Procedures previously described were used for the measurement of comparative cell growth on agar plates buffered at pH 9.0 (4, 12).

RESULTS

A Gene Involved in a Novel HCO_3^- Transport System—To isolate novel mutants impaired in HCO_3^- uptake in *Synechocystis* 6803 and identify the relevant genes, it was essential to use strains defective in both CO_2 uptake and in the *cmp* operon that encodes an ABC-type HCO_3^- transporter (5). The B1 strain, impaired in *ndhB*, was selected as a proper host since it is unable to take up CO_2 and does not grow at pH 7.0 under air level of CO_2 (12, 13). On the

other hand, this mutant exhibited normal HCO_3^--transport activity and could grow like the WT in air at pH 9.0 (12), conditions where inorganic carbon (Ci) is mainly supplied by HCO_3^- transport. Inactivation of the *cmp* operon in the B1 mutant did not change its growth characteristics at pH 9.0, under either high or low levels of CO_2 (not shown), suggesting that HCO_3^- uptake capability was not impaired. We transformed the double mutant B1/*ΔcmpA* with a transposon-bearing inactivation library (3) and isolated four mutants defective in their ability to grow at pH 9.0 under air level of CO_2 and unable to take up HCO_3^-. All these mutants (NB-3, 9, 10 and 48) had Cm^R cassettes at various sites within a single gene, *slr1512* (designated *sbtA*, Fig. 1A). WT *Synechocystis* 6803 and the *ΔndhD3*, *ΔndhD4*, *ΔndhD3/ndhD4* (hereafter *ΔndhD3/D4*) and *ΔndhD3/D4/cmpA* mutants were transformed with the genomic DNA from strain NB-3 in order to interrupt their *sbtA*. As shown in Fig. 1B, all the mutants obtained, with the exception of *ΔndhD3/D4/sbtA* and *ΔndhD3/D4/cmpA/sbtA* (not shown), grew like the WT at pH 9.0 in air and in 3% CO_2. Inactivation of *sbtA* and/or *cmpA* in WT cells had no effect on their growth (not shown) presumably since the mutants were able to take up sufficient CO_2 to support their growth. Similarly, disruption of *sbtA* in the single *ΔndhD3* or *ΔndhD4* mutants, which are able to take up CO_2 either by the constitutive or by the inducible systems (3,4,12), had no effect on their growth (upper panel, Fig. 1B). It is most likely that the ability to take up HCO_3^- enabled growth of the *ΔndhD3/D4* mutant at alkaline pH and air level of CO_2. However, inactivation of *sbtA* in this double mutant resulted in the loss of its ability to grow under low CO_2 even at pH 9 (Fig. 1B). These results suggested that the gene product of *sbtA* is involved in HCO_3^- transport and that its activity could support growth of the *ΔndhD3/D4* mutant, particularly at pH 9.0. In contrast to the *ΔndhD3/D4/sbtA*

mutant, inactivation of *cmpA* in the *ΔndhD3/D4* strain scarcely affected its growth (Fig. 1B). These results indicated that the contribution of the Cmp-dependent HCO_3^- transport to the growth of *Synechocystis* 6803 is negligible. All mutants examined, with the exception of *ΔndhD3/D4/sbtA* (lower panel in Fig. 1B) and *ΔndhD3/D4/sbtA/cmpA* (not shown) grew like the WT on agar plates under 3% CO_2. The latter mutants could grow like the WT in liquid medium at pH 9.0 in 3% CO_2 in air (v/v) but not in air alone (Fig. 1C).

Inactivation of the *slr1513* gene, located downstream of *sbtA* (Fig. 1A), within the *ΔndhD3/D4* mutant had no effect on growth performance (not shown). This result ruled out a possible pleiotrophic effect due to interruption of *sbtA*. The possibility that *sbtA* encodes a novel HCO_3^- transporter was examined further by measuring the activity of HCO_3^- transport and the expression of *sbtA* in the WT and the mutants (Figs. 2 and 3).

A Low-CO_2 Inducible, Na^+-Dependent HCO_3^- Transport is Mediated by SbtA—Figure 2A shows the amounts of HCO_3^- taken up by WT and various mutants during 15 sec incubation with 400 μM HCO_3^-. There was no significant difference between the amounts of HCO_3^- taken up by the WT and by mutants *ΔndhD3/D4*, *ΔndhD3/D4/cmpA* (a, b and c). HCO_3^- uptake by *ΔndhD3/D4/cmpA* was about six times higher in light than in darkness (c and c'). Inactivation of *sbtA* in *ΔndhD3/D4* severely depressed the rate of HCO_3^- uptake (d and d'); disruption of *cmpA* in *ΔndhD3/D4/sbtA* reduced the HCO_3^--transport activity somewhat further (e and e'). The low level of HCO_3^- uptake observed in *ΔndhD3/D4//sbtA/cmpA* most likely reflected non-specific adherence of ^{14}Ci to the cells since light did not stimulate this apparent uptake. These data indicated that the SbtA-mediated system plays the major role in HCO_3^- uptake in *Synechocystis* 6803 and that the contribution of the Cmp-mediated HCO_3^- transport was very

small. This is in agreement with the ability of *ΔndhD3/D4/cmpA,* but not *ΔndhD3/D4/sbtA* and *ΔndhD3/D4/cmpA/sbtA*, to grow under low [CO_2] (Fig. 1B).

A small amount of transcript originating from *sbtA* was detected in the WT and *ΔndhD3/D4* mutant cells of *Synechocystis* 6803 grown under 3% CO_2 (H-cells, lanes a and d for *sbtA* in Fig. 3) but the transcript abundance increased significantly within 2 to 6 hrs of exposure to air level of CO_2 (lanes b, c, e and f for *sbtA*). This data indicated that expression of *sbtA* was induced by low CO_2 in the WT and *ΔndhD3/D*4 mutant, in agreement with the large rise in HCO_3^- transport activity in cells acclimated to air level of CO_2 (c, f and i for L-cells *versus* h for H-cells in Fig. 2). A transcript of *cmpA* was not detectable in H-cells of the WT and *ΔndhD3/D4* mutant (Fig. 3, lanes a and d for *cmpA*) but was detected in the WT cells acclimated to air for 6 hrs (lane c for *cmpA*). The *cmpA* transcript was barely detectable in the mutant even after 6 hrs of acclimation to air (lane f for *cmpA*). This may explain the very low activity of the Cmp-dependent HCO_3^- transport in the *ΔndhD3/D4/sbtA* mutant (Fig. 2, d). The SbtA-dependent HCO_3^- uptake was strongly affected by the ambient pH level. At pHs 8.0 (Fig. 2, j) and 7.0 (k), HCO_3^- uptake was about 50 and 20 %, respectively, that observed at pH 9.0 (i). SbtA-mediated HCO_3^- transport was almost completely abolished when NaCl in the medium was replaced with KCl (g), indicating that HCO_3^- transport is specifically dependent on the presence of Na^+ ions. Figures 4A and 4B show the dependency of the SbtA-mediated HCO_3^- transport in the *ΔndhD3/D4/cmpA* mutant to HCO_3^- and Na^+ concentrations, respectively. Maximal rate of HCO_3^- uptake was reached at 100 μM HCO_3^- and the $K_{1/2}(HCO_3^-)$ value was about 16 μM (open circles in Fig. 4A). Photosynthetic O_2 evolution displayed a similar dependency on external [HCO_3^-] (closed circles), suggesting that in this

mutant photosynthesis was rate-limited by the SbtA-mediated HCO_3^- transport. Dependency of the SbtA-mediated HCO_3^- transport on ambient [Na^+] was further supported by the nature of the curve relating HCO_3^- uptake to [Na^+] (Fig. 4B). Maximal HCO_3^- uptake was attained at 6 mM Na^+ and the concentration of Na^+ essential to support half maximal HCO_3^- transport was about 1 mM (Fig. 4B). These results are in general agreement with an earlier report (10) on the response of HCO_3^- uptake in *Synechocystis* 6803 to the presence of Na^+. The higher maximal rate of HCO_3^- uptake observed before was, most likely, due to simultaneous uptake of CO_2 in WT where both the constitutive and the inducible CO_2-uptake systems (3) are functional. Furthermore, analysis of CO_2 uptake by mutant *ΔcmpA/sbtA* (unable to take up HCO_3^-, Fig. 4C) showed that it increased linearly with the ambient [HCO_3^-] well above the amount of CO_2 that could be produced spontaneously from HCO_3^- at pH 9.0 (broken line). These data clearly indicated that conversion of HCO_3^- to CO_2 at the cell surface is faster than expected from physicochemical considerations based on the concentration of HCO_3^- and pH in the bulk medium. Formation of CO_2 may be catalyzed by a periplasmic-located carbonic anhydrase (18) or accelerated by light-dependent proton extrusion that could acidify the periplasmic space (8, 19, 20).

NtpJ is Involved in HCO_3^- Transport—The specific dependence of the SbtA-mediated HCO_3^- transport on [Na^+] (Figs. 2B and 4B) recalls earlier studies (7, 9, 10, 21) where various possibilities were raised to explain the role of Na^+. If the $\Delta\mu Na^+$ across the cytoplasmic membrane is essential for the operation of the SbtA-mediated HCO_3^- transport, inactivation of components involved in Na^+ extrusion (primary Na^+ or Na^+/H^+ pumps) should affect the HCO_3^- uptake and growth of a mutant unable to utilize CO_2 such as *ΔndhD3/D4* (Fig. 5).

Synechocystis 6803 can grow under a relatively high [NaCl] even exceeding 0.5 M (22). Inactivation of *slr1509* (*ntpJ*), encoding a protein that belongs to a Na^+-transporter family (http://motif.genome.ad.jp/), barely affected the growth of *Synechocystis* 6803 in BG11 medium at pH 9.0 in air, but growth was severely depressed when [NaCl] was raised above 100 mM (Fig. 5A). These results suggested that NtpJ could be involved in Na^+ extrusion and that failure of the mutant to extrude Na^+ abolished its growth at elevated [NaCl]. In contrast to the WT, inactivation of *ntpJ* completely abolished growth of the *ΔndhD3/D4* mutant even in BG11 medium in air (Fig. 5B). On the other hand, under 3% CO_2, the *ΔndhD3/D4/ntpJ* mutant grew almost like the WT (Fig. 5B). These results suggested involvement of NtpJ in the supply of Ci for growth. This was confirmed by measuring the uptake of HCO_3^- uptake by this mutant (Fig. 5C). The HCO_3^--transport activity in the *ΔndhD3/D4/ntpJ* mutant was only about one third of that in the *ΔndhD3/D4* mutant (Fig. 5C) and became much lower during longer exposure of the mutant to light. These results support the notion that NtpJ is a subunit of a Na^+ extrusion pump essential for the SbtA-mediated HCO_3^- transport.

Phylogenetic Analysis of SbtA and NtpJ in Cyanobacteria—Homologues of SbtA have been identified in *Synechococcus* sp. PCC 6301 (Dr. M. Sugita, personal communication), *Synechococcus* sp. PCC 7002 (Drs. J. Zhao and D. Bryant, personal communication), *Anabaena* PCC 7120 (http://www.kazusa.or.jp/cyano/), *Nostoc punctiforme, P. marinus* strains MED4 and MIT9313 (http://www.jgi.doe.gov/tempweb/JGI_microbial_/html/index.html) and in the non-photosynthetic bacteria *Mycobacterium tuberculosis* (23), *Caulobacter crescentus* (24) and *Bacillus halodurans* (25). The phylogenetic tree (Fig. 6A) pointed to two types of SbtA in cyanobacteria, one consisting of 370-374 and the other of 324-339 amino acids.

Anabaena possesses both types of SbtA. The sequence homology between the two types of SbtA is relatively weak but analyses of hydrophobicity profiles indicated that both types contains 10 membrane-spanning domains that are structurally highly conserved (Fig. 7). Search for specific motifs with the aid of TargetP program (26) identified a signal polypeptide sequence in the N-terminal region of both types of SbtA, likely to target them to the cell exterior and/or the thylakoid lumen. Presently, the exact location of the SbtA is not known, but based on the data presented here and its involvement in Na^+ exchange, it is most likely located on the cytoplasmic membrane.

All the cyanobacterial strains investigated possess the NtpJ essential for the operation of the SbtA-mediated HCO_3^- transport. The phylogenetic tree of NtpJ indicated two types of proteins, one present in both strains of *P. marinus* and the other in the other organisms (Fig. 6B).

DISCUSSION

Four Systems for Ci Acquisition in Synechocystis 6803—*Synechocystis* 6803 appears to possess four different systems for Ci acquisition. Two of them, recently identified, are engaged in CO_2 uptake (3). The other two, involved in HCO_3^- transport, are the ABC-type transporter encoded by *cmpA-D* (5) and the SbtA-mediated system identified here. It was essential to inactivate both CO_2-uptake systems to recover the *ΔsbtA* mutants since presence of either of them enabled photoautotrophic growth even at pH 9.0 in air (Fig. 1B). Measurements of growth and of HCO_3^- uptake (Figs. 1 and 2) indicated that SbtA plays the central role in HCO_3^- transport in *Synechocystis* 6803 and that the contribution of the CmpABCD-mediated HCO_3^-

transport is negligible, also in mutant *ΔndhD3/D4*. Furthermore, lack of HCO_3^- uptake in the *ΔndhD3/D4/sbtA/cmpA* mutant ruled out the possibility that Slr1515 (homolog of IctB from *Synechococcus* 7942, ref. 27) is an independent HCO_3^- transporter in *Synechocystis* 6803. The role of Slr1515 (IctB) in intracellular HCO_3^- accumulation in cyanobacteria is not known and we were unable to inactivate *slr1515* in *Synechocystis* 6803. The inability to inactivate *ictB* (or its homologue, *slr1515*) suggests that its gene product plays a very important role. Based upon the observations presented here, one might expect that this protein act downstream from SbtA/CmpA/NdhD3/NdhD4. Enhancement of the expression of *sbtA* by low CO_2 (Fig. 3) was in agreement with the considerable rise in HCO_3^- transport capability in cells grown under these conditions (Fig. 2).

The Nature and Mode of Energization of the SbtA-Mediated HCO_3^- Transport—Data presented here may help to identify the primary pump involved in the SbtA-mediated active HCO_3^- transport. SbtA does not possess an ATP-binding domain. It is therefore unlikely that ATP directly fuels it. SbtA-mediated HCO_3^- transport was strongly and specifically dependent on the presence of Na^+ ions (Figs. 2B and 4B) and NtpJ was essential for both the growth of *Synechocystis* 6803 in the presence of elevated [Na^+] and for HCO_3^- transport (Fig. 5). These data are consistent with the suggestion that SbtA is a component of a Na^+/HCO_3^- symporter that drives the HCO_3^- transport secondary to a primary Na^+ pump (7, 9, 10). The latter is essential to establish the $\Delta\mu Na^+$ for active HCO_3^- accumulation. The nature of this primary sodium extrusion pump (28) is not known but NtpJ is likely to be involved. Measurements of the $\Delta\mu Na^+$ value and of the Na^+ flux across the cytoplasmic membrane of *Synechocystis* 6803, as affected by [Na^+], [HCO_3^-] and pH, are not available. In a detailed study, Ritchie et al (21)

measured some of these parameters in *Synechococcus* 7942. They concluded that $\Delta\mu Na^+$ would be large enough to drive HCO_3^- uptake if the stoichiometry of Na^+:HCO_3^- is 2 or 3:1. Since the internal HCO_3^- pool in *Synechocystis* 6803 is 8 to 10-fold smaller than in *Synechococcus* 7942 (10), a smaller $\Delta\mu Na^+$ would suffice. Measurements of $^{22}Na^+$ uptake in *Synechococcus* 7942 showed large enhancement by the presence of HCO_3^- (8). On the other hand, Ritchie et al (21) concluded that the Na^+ flux was not sufficient to support the rate of photosynthesis (thought to be supported solely by HCO_3^- transport). However, photosynthesis in both *Synechocystis* 6803 and *Synechococcus* 7942 is largely supported by CO_2 uptake, even at high external pH.

The alternative possibility that HCO_3^- transport is energized by the $\Delta\mu Na^+$ generated by a Na^+/H^+ antiporter, secondary to H^+-ATPase (29), is unlikely. SbtA-mediated HCO_3^- transport activity was highest at pH 9.0 and lowest at pH 7.0 whereas the $\Delta\mu H^+$ in cyanobacteria declines with rising pH. At alkaline pH such as 9.0, $\Delta\mu H^+$ would not suffice to drive HCO_3^- uptake (8, 21). We cannot dismiss the possibility that Na^+ binds to the HCO_3^- carrier and alters its kinetic parameters (7, 10). However, the fact that a *ΔntpJ* mutant was impaired in both the ability to grow under high Na^+ and take up HCO_3^- lends support to the possibility that NtpJ is involved in Na^+ extrusion rather than in the affinity of the HCO_3^- carrier for its substrate. This is further supported by the suggestion that NtpJ belongs to a Na^+-transporter family (http://motif.genome.ad.jp/) and it is homologues to a subunit of HKT1 in *Arabidopsis thaliana* that mediates Na^+ transport (30). We suggest that it is most plausible that the SbtA-mediated HCO_3^- transport is energized by a primary Na^+ pump. Detailed studies on NtpJ and homologues of other subunits of HKT1 are being performed to assess their role in

Na^+ extrusion.

Comparative Sequence Analysis of SbtA—All the cyanobacterial strains examined, with the exception of *P. marinus* strains, possesses genes involved in CO_2 uptake (3). Phylogenetic analysis indicated that two types of SbtA exist in cyanobacteria; one in *Synechocystis* 6803, *Synechococcus* sp. PCC 6301 and *Synechococcus* sp. PCC 7002, and the other in *N. punctiforme* and *P. marinus* strains MED4 and MIT9313 (Fig. 5A). *Anabaena* sp. strain PCC 7120 possesses both types of SbtA. *N. punctiforme* is evolutionary very close to *Anabaena* PCC 7120. Therefore, it is likely that the second type of SbtA is located the the genomic regions of *Nostoc* yet to be revealed. We may conclude that *P. marinus* strains acquire Ci by HCO_3^- transport and that the SbtA-mediated HCO_3^- transport plays a crucial role in the acquisition of Ci either when the supply of CO_2 is limited or in organisms such as *P. marinus* strains that do not possess a CO_2-uptake system. The *Prochlorococcus* group is thought to be the most abundant photosynthetic organism on the planet (31), and is responsible for a significant fraction of CO_2 fixation in the oceans. The present study suggests a crucial role of the SbtA-mediated HCO_3^- transport in the acquisition of Ci by *P. marinus* and, therefore, for carbon fixation in the oceans.

Bicarbonate transporters are the principal regulators of pH in animal cells and have a vital role in acid-base movement. The functional family of HCO_3^- transporters includes Cl^- /HCO_3^- exchangers, three Na^+/HCO_3^- co-transporters and K^+/HCO_3^- co-transporter (32, 33). These transporters are much larger than SbtA and there was no similarity in amino-acid sequences between SbtA and mammalian-type HCO_3^- transporters.

FOOTNOTES

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[1]The abbreviations used are: CCM, CO_2-concentrating mechanism; Chl, chlorophyll; Ci, inorganic carbon; H-cells, cells grown under 3% (v/v) CO_2 in air; L-cells, cells acclimated to air for 18 hrs in the light; WT, wild type.

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Figure legends

Fig. 1. The structure of the *sbtA (slr1512)* region and the Cm^R cassette tags and Hyg^R cassette interrupting the genes (A), the growth of WT and mutants on agar plates (B) and of WT and *ΔndhD3/D4/cmpA/sbtA* mutant in liquid (C) at pH 9.0 under air or air enriched with 3% CO_2 (v/v). (A) The positions of the Cm^R cassette in *sbtA* are 109, 155, 441 and 1056 base pairs down-stream of the initiation codon of *sbtA* for NB-10, NB-48, NB-3 and NB-9, respectively. A fragment between 98 and 142 base pairs downstream of the initiation codon of *slr1513* was replaced with a hygromycin resistance cassette (Hyg^R). The horizontal arrows indicate the direction of the cassettes. (B) Two µl of cell suspensions with densities corresponding to OD_{730nm} values of 0.1 (upper rows of panels in B), 0. 01 (middle rows) and 0.001 (lower rows) were spotted on agar plates containing medium BG11 buffered at pH 9.0. The plates were incubated under 3% CO_2 in air (v/v) or air alone for 5 days at 50 µmol photons.$m^{-2}s^{1}$. (C) The growth of WT (triangles) and *ΔndhD3/D4/cmpA/sbtA* mutant (circles) in BG11 (pH 9.0) under 3% CO_2 in air (v/v) (H, open symbols) or air (L, closed symbols).

Fig. 2. The uptake of HCO_3^- by the WT and various mutants (A) and by the *ΔndhD3/D4/cmpA* mutant (B). Unless otherwise stated, cells grown at 3% CO_2 in air (v/v) were aerated with air overnight and were suspended in the assay buffer of pH 9 containing 15 mM NaCl and 400 µM HCO_3^-. Cells were suspended in the assay buffer of pH 8 and 7 for j and k, respectively, and in the assay buffer of pH 9.0 in

which NaCl was replaced with KCl for g. H-cells were used for h. Cells were incubated for 15 sec either in light (a - k) or in darkness (c' – e'). Vertical bars indicate standard deviations (n = 5).

Fig. 3. The transcript levels of *sbtA* and *cmpA* in the WT (a, b, c) and *ΔndhD3/D4* mutant (d, e, f). Transcript abundance in H-cells (a, d) or H-cells adapted to air for 2 (b, e) and 6 hrs (c, f) was determined by the RT-PCR method (15). The transcript levels of RNaseP (17) in each sample are shown as a control. The absence of contamination of DNA was confirmed by PCR without RTase reaction.

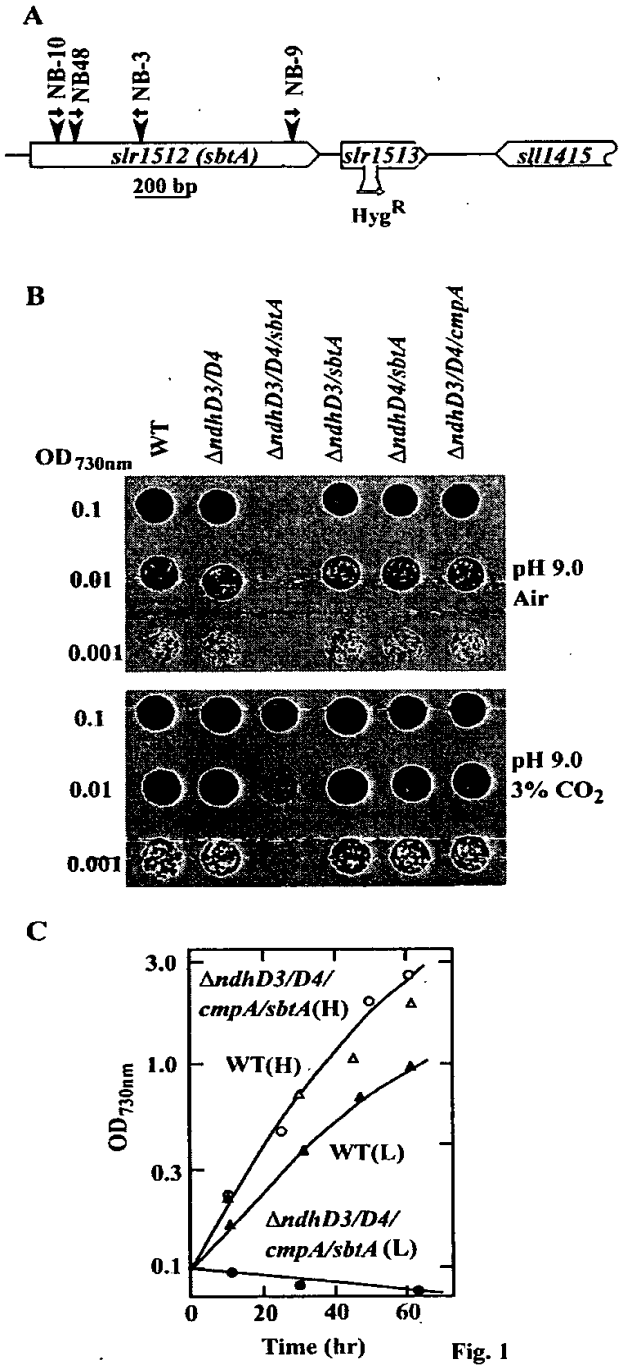
Fig. 4. The uptake of HCO_3^- by the *ΔndhD3/D4/cmpA* (A, B) and *ΔcmpA/sbtA* (C) strains as a function of HCO_3^- (A, C) and Na^+ (B) concentrations. HCO_3^- uptake was measured in the medium of pH 9.0 containing 15 mM NaCl for (A) and (C) and 15 mM KCl/400 μM HCO_3^- for (B) and various concentrations of HCO_3^- for (A) and (C) and NaCl for (B). The closed triangles in (C) indicate the values obtained for the *ΔndhD3/D4/cmpA/sbtA* mutant. Vertical bars indicate standard deviations (n = 5). O_2 evolution was measured with cells suspended in BG-11 medium buffered at pH 9.0 containing 15 mM NaCl.

Fig. 5. Effect of inactivation of *ntpJ* in WT and in the *ΔndhD3/D4* mutant on their growth and HCO_3^- uptake activity. (A) Growth rates of the WT and *ΔntpJ* strains in BG-11 medium, pH 8.0, containing various concentrations of NaCl under aeration

with 3% CO_2 in air (v/v). (B) Growth of the WT, *ΔntpJ* and *ΔndhD3/D4/ntpJ* strains on agar plates buffered at pH 9.0 under the conditions described in the legend for Fig. 1. (C) The HCO_3^--transport activity of low CO_2-adapted cells of the *ΔndhD3/D4* and *ΔndhD3/D4/ntpJ* mutants suspended in the assay buffer of pH 9 containing 15 mM NaCl and 400 μM HCO_3^-. Vertical bars indicate standard deviations (n = 5).

Fig. 6. Phylogenetic trees of SbtA (A) and NtpJ (B). Multiple sequence alignment was performed using the CLUSTAL program (34). Syn6803, *Synechocystis* 6803; Syn6301, *Synechococcus* sp. strain PCC 6301; Syn7002, *Synechococcus* sp. strain PCC 7002; Ana, *Anabaena* sp. strain PCC 7120; Nos, *Nostoc punctiforme*; ProMED, *P. marinus* MED4; ProMIT, *P. marinus* MIT9313; Bacillus, *Bacillus halodurans*; Caulo, *Caulobacter crescentus*; Myco, *Mycobacterium tuberculosis*.

Fig. 7. The hydropathy profiles of two types of SbtA. The profiles were determined by the method of Kyte and Doolittle (35) using a window size of 17 amino acids.


A
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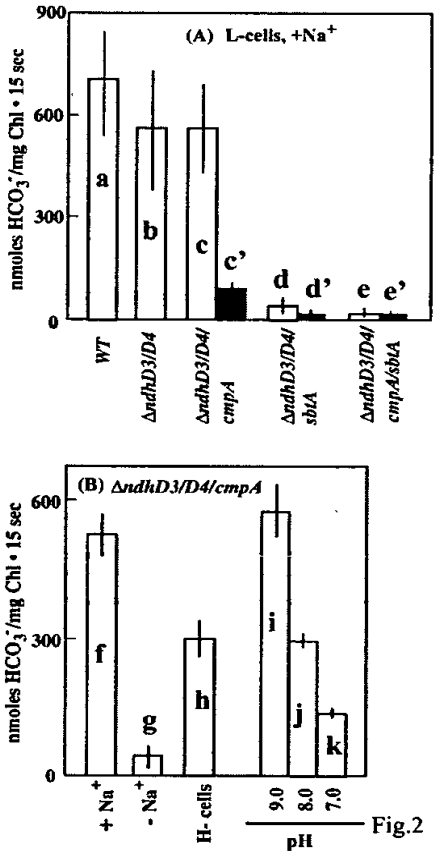
NB48
NB-3
NB-9
slr1512 (sbtA)
slr1513
sll1415
200 bp
Hyg^R
B
WT
ΔndhD3/D4
ΔndhD3/D4/sbtA
ΔndhD3/sbtA
ΔndhD4/sbtA
ΔndhD3/D4/cmpA
OD730nm
0.1
0.01
0.001
pH 9.0
Air
pH 9.0
3% CO2
C
3.0
1.0
0.3
0.1
OD730nm
ΔndhD3/D4/
cmpA/sbtA(H)
WT(H)
WT(L)
ΔndhD3/D4/
cmpA/sbtA (L)
0
20
40
60
Time (hr)

Fig. 1


(A) L-cells, +Na+
nmoles HCO3-/mg Chl • 15 sec
900
600
300
0
a
b
c
c'
d
d'
e
e'
WT
ΔndhD3/D4
ΔndhD3/D4/cmpA
ΔndhD3/D4/sbtA
ΔndhD3/D4/cmpA/sbtA
(B) ΔndhD3/D4/cmpA
nmoles HCO3-/mg Chl • 15 sec
600
300
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+ Na+
- Na+
H- cells
9.0
8.0
7.0
pH

Fig.2

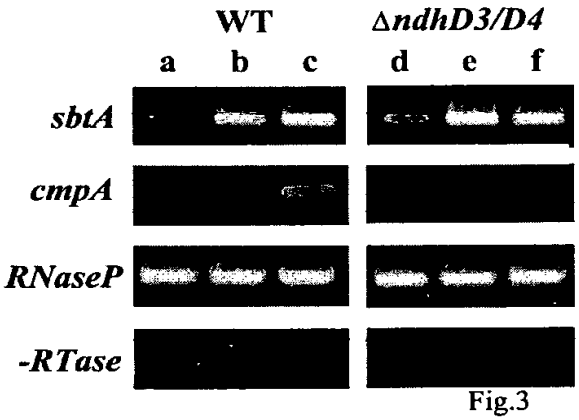


Fig.3

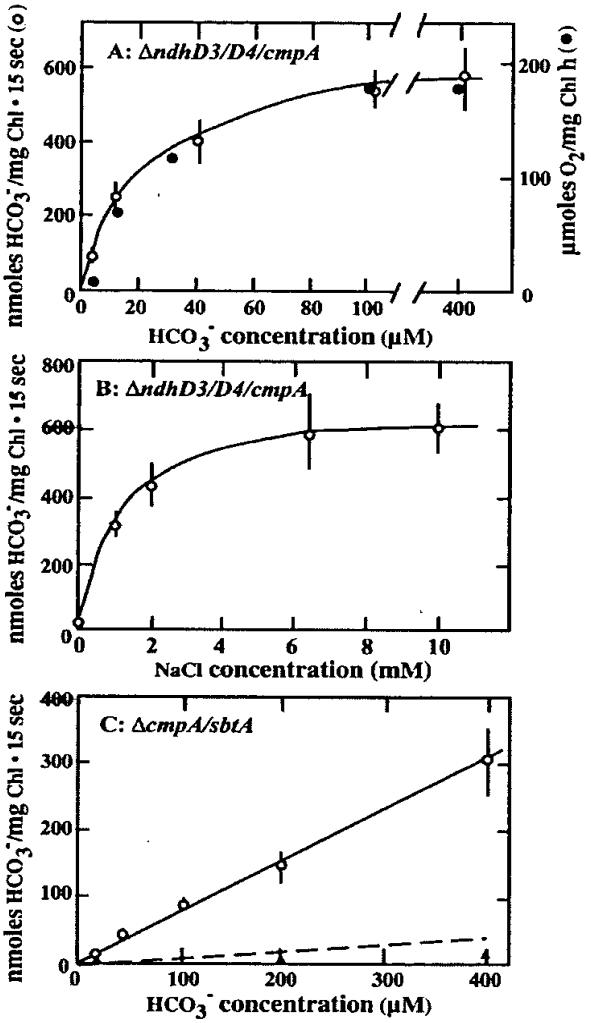

A: ΔndhD3/D4/cmpA
nmoles HCO3-/mg Chl • 15 sec (o)
µmoles O2/mg Chl h (•)
HCO3- concentration (µM)
B: ΔndhD3/D4/cmpA
nmoles HCO3-/mg Chl • 15 sec
NaCl concentration (mM)
C: ΔcmpA/sbtA
nmoles HCO3-/mg Chl • 15 sec
HCO3- concentration (µM)

Figure 4

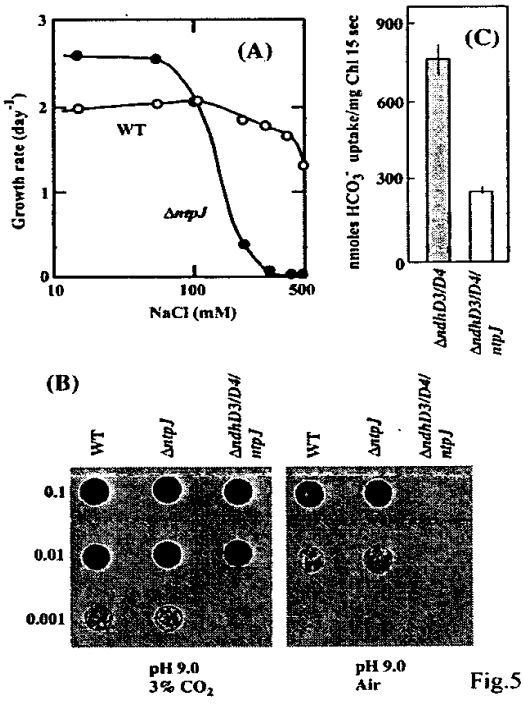
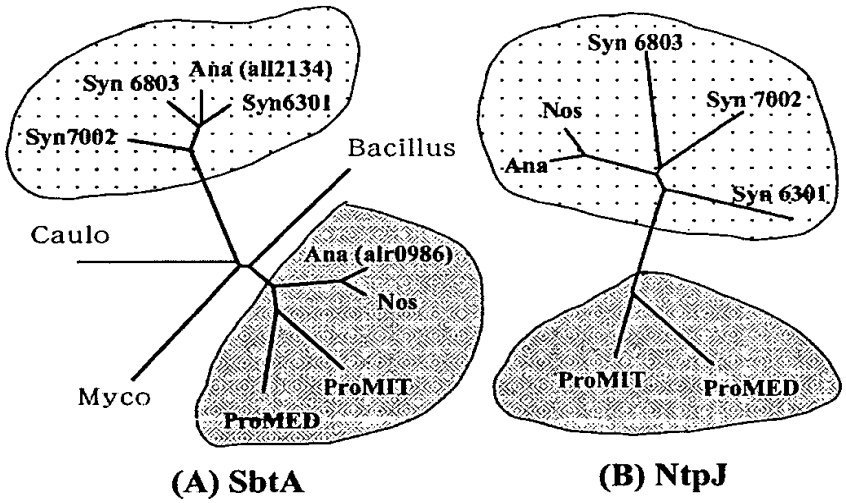

(A)
Growth rate (day^{-1})
WT
ΔntpJ
NaCl (mM)
10
100
500
(C)
nmoles HCO_3^- uptake/mg Chl 15 sec
ΔndhD3/D4
ΔndhD3/D4/ ntpJ
(B)
WT
ΔntpJ
ΔndhD3/D4/ ntpJ
0.1
0.01
0.001
pH 9.0
3% CO_2
pH 9.0
Air

Fig.5



(A) SbtA **(B) NtpJ**

Fig. 6

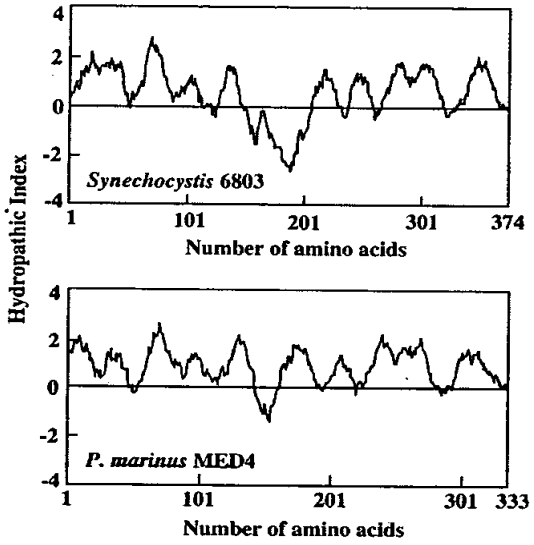

Hydropathic Index
4
2
0
-2
-4
Synechocystis 6803
1
101
201
301
374
Number of amino acids
4
2
0
-2
-4
P. marinus MED4
1
101
201
301
333
Number of amino acids

Fig. 7

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Enhanced photosynthesis and growth of transgenic plants that express *ictB*, a gene involved in HCO_3^- accumulation in cyanobacteria

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Summary

Transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* plants that express *ictB*, a gene involved in HCO_3^- accumulation within the cyanobacterium *Synechococcus* sp. PCC 7942, exhibited significantly faster photosynthetic rates than the wild-types under limiting but not under saturating CO_2 concentrations. Under conditions of low relative humidity, growth of the transgenic *A. thaliana* plants was considerably faster than the wild-type. This enhancement of growth was not observed under humid conditions. There was no difference in the amount of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) detected in the wild-types and their respective transgenic plants. Following activation *in vitro*, the activities of RubisCO from either low- or high-humidity-grown transgenic plants were similar to those observed in the wild-types. In contrast, the *in vivo* RubisCO activity, i.e. without prior activation, in plants grown under low humidity was considerably higher in *ictB*-expressing plants than in their wild-types. The CO_2 compensation point in the transgenic plants that express *ictB* was lower than in the wild-types, suggesting that the concentration of CO_2 in close proximity to RubisCO was higher. This may explain the higher activation level of RubisCO and enhanced photosynthetic activities and growth in the transgenic plants. These data indicated a potential use of *ictB* for the stimulation of crop yield.

Introduction

Plants that belong to the physiological C4 type or the Crassulacean acid metabolism groups, as well as many photosynthetic micro-organisms, possess various types of CO_2 concentrating mechanisms (Cushman and Bohnert, 2000; Hatch, 1992; Kaplan and Reinhold, 1999). These mechanisms enable them to raise the concentration of CO_2 in close proximity to ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) and hence overcome, at least partly, the low affinity of the enzyme for CO_2. In contrast, the majority of higher plants that belong to the C_3 group, including most crop plants, do not possess this ability. Therefore, under many environmental conditions plant photosynthesis is rate-limited by the concentration of CO_2 at the carboxylation site and/or by the activity of RubisCO. Attempts are being made to raise the apparent photosynthetic affinity of C_3 plants for CO_2 by various biotechnological approaches. These include a search for a RubisCO that exhibits an elevated specificity for CO_2 among natural photosynthetic populations (Uemura *et al.*, 1997; Horken and Tabita, 1999; Tabita, 1999); site directed modifications of the enzyme (Cleland *et al.*, 1998; Kostov *et al.*, 1997; Ramage *et al.*, 1998; Spreitzer and Salvucci, 2002) and expression of genes involved in C_4 metabolism within C_3 plants (Ku *et al.*, 1999; Matsuoka *et al.*, 2001; Nomura *et al.*, 2000; Surridge, 2002). Characterization of a high-CO_2-requiring mutant of the cyanobacterium *Synechococcus* sp. strain PCC 7942 (hereafter *Synechococcus* PCC 7942) implicated *ictB* as a gene involved in inorganic carbon accumulation in this organism (Bonfil *et al.*, 1998). IctB is highly conserved among cyanobacteria but its exact role is not known, since it was not possible to directly inactivate

it or its homologue *slr1515* in *Synechocystis* sp. strain PCC 6803.

Ability to stimulate photosynthesis and growth of tobacco by the expression of a single cyanobacterial gene encoding fructose-1,6/sedoheptulose-1,7-bisphospate phosphatase was recently demonstrated (Miyagawa *et al.*, 2001). Apparently, the level of intermediates of the Calvin cycle was raised in the transgenic tobacco plants suggesting that, under the conditions of their experiments, photosynthesis was rate-limited by the level of ribulose 1,5-bisphosphate. In this study we show that expression of *ictB* from *Synechococcus* PCC 7942 enhanced photosynthesis and growth in C_3 plants due to a higher internal CO_2 concentration at the site of RubisCO and consequently higher enzyme activity in the transgenic plants.

Results

Expression of *ictB*

Transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* (tobacco) plants bearing *ictB* were raised after transformation with *Agrobacterium* (see Experimental procedures). Northern analyses performed on total RNA isolated from the wild-type and kanamycin-resistant transformed lines demonstrated that three transgenic *Arabidopsis* (A, B and C) and five (1, 3, 4, 8 and 11) tobacco plants expressed *ictB* to different extents (Figure 1). We did not detect a transcript of *ictB* in the wild-type plants. Southern analyses of DNA from the transgenic *Arabidopsis* and tobacco plants indicated that the construct bearing *ictB* was inserted in different sites within their genomes (not shown).

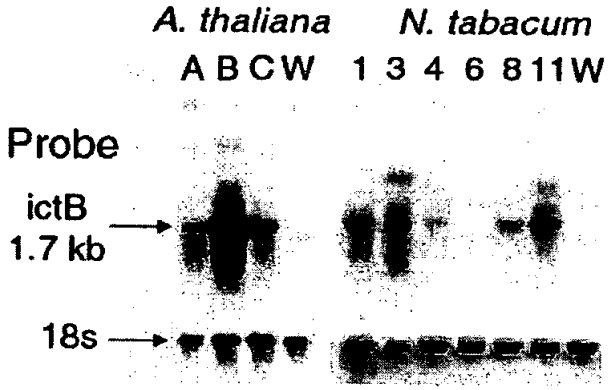

Figure 1 Northern blots of RNA isolated from transgenic and wild-type (W) *Arabidopsis* and tobacco plants hybridized to both *ictB* and 18S rDNA probes. 30 µg RNA was loaded in each lane. RNA was isolated as described in the Experimental procedures. Northern blot analyses on 1% agarose gels, was done as described by Mittler and Zilinskas (1992).

Photosynthetic performance

In *Synechococcus* PCC 7942, *ictB* is involved in the accumulation of inorganic carbon, and a mutant impaired in this gene demanded a high CO_2 concentration for photosynthesis. Therefore, we examined the rate of photosynthesis in the wild-types and the transgenic plants as it was affected by intercellular CO_2 concentration. Generally, despite the fact that the expression of *ictB* varied markedly between transgenic plants, in both *Arabidopsis* and tobacco (Figure 1), there was hardly any difference between their photosynthetic performances. Plants that expressed *ictB* showed similar photosynthetic characteristics, which differed markedly from those that did not express this gene. At saturating CO_2 levels, the photosynthetic rates of transgenic tobacco (Figure 2A) and *Arabidopsis* plants (Figure 2B) were similar to those found in their wild-types. This suggested that the ability to perform maximal photosynthesis was not affected by the expression of *ictB*. In contrast, under limiting intercellular CO_2 concentrations, the transgenic tobacco lines 1, 3 and 11 (Figure 2A) and *Arabidopsis* plants A, B (Figure 2B) and C (not shown) showed significantly higher photosynthetic rates than the wild-types. Notably, some of the transgenic, kanamycin-resistant plants which did not express *ictB* (cf. tobacco plant number 6, Figure 2A), exhibited either similar or sometimes even slightly lower photosynthetic rates than the respective wild-type. Stomatal conductances, measured by the Li-Cor 6400 or the Delta-T porometer (model MK3, UK), were lower in plants grown under the dry conditions but did not differ significantly between the wild-types and the transgenic plants (Table 1). These data confirmed that the higher photosynthetic rate at limiting intercellular CO_2

Table 1 Stomatal conductance in wild-type (WT) and transgenic *Arabidopsis* and tobacco plants. Plants grown under humid (70–75% relative humidity) or dry (25–30% humidity) conditions were used in these experiments

Plant	High humidity	Low humidity
Tobacco WT	686.8 ± 3.6	196.0 ± 1.2
Tobacco Plant 3	682.6 ± 4.5	196.7 ± 1.6
Tobacco Plant 11	684.3 ± 3.1	196.2 ± 1.2
Arabidopsis WT	597.9 ± 3.5	209.1 ± 1.3
Arabidopsis Plant A	598.4 ± 3.1	209.7 ± 1.7
Arabidopsis Plant B	599.5 ± 3.2	208.9 ± 1.3

The data are presented in mmole/m²/s, as the average ± SE, n = 18.

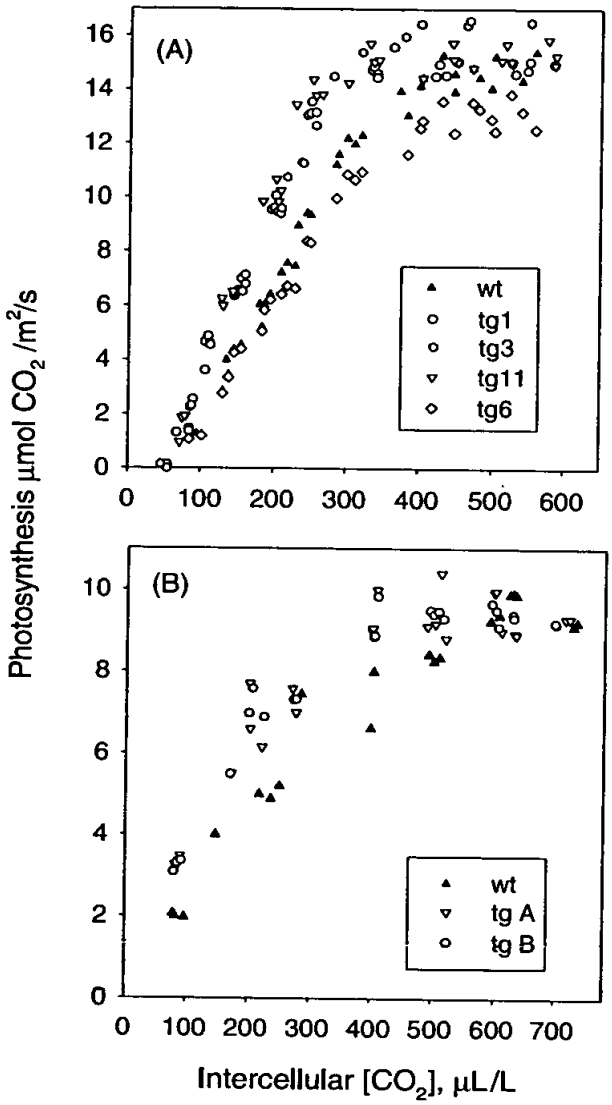

Figure 2 The rate of photosynthesis as affected by the intercellular concentration of CO_2 in wild-type (wt) and transgenic (tg) tobacco (A) and *Arabidopsis* (B) plants. The plants were grown under dry conditions. $n = 8$.

concentrations did not result from a higher conductance of CO_2 in the transgenic plants.

CO_2 compensation point

Involvement of IctB in the ability of *Synechococcus* PCC 7942 to accumulate HCO_3^- internally (Bonfil *et al.*, 1998) raised the possibility that the higher photosynthetic and RubisCO activities (see below) at limiting CO_2 concentrations in the transgenic plants were due to an elevated CO_2 concentration in close proximity to RubisCO. Should this be the case, it would

Table 2 The CO_2 compensation points in wild-type (WT) and transgenic *Arabidopsis* and tobacco plants. The compensation points were deduced from measurements of the rate of CO_2 exchange over a range of CO_2 concentrations of 0–150 µL/L as described in Experimental procedures

Plant	CO_2 compensation (µL/L)
Arabidopsis Plant A	39.2 ± 1.0
Arabidopsis Plant B	41.0 ± 1.1
Arabidopsis WT	46.1 ± 1.1
Tobacco Plant 3	47.1 ± 1.4
Tobacco Plant 11	48.0 ± 1.6
Tobacco Wild-type	56.9 ± 1.6

The data are presented as the average ± SE, $n = 18$.

be expected to lower the CO_2 compensation point (i.e. the ambient CO_2 concentration, where the net CO_2 exchange is zero since CO_2 uptake in photosynthesis is equal to the sum of respiratory and photorespiratory CO_2 efflux). We examined the CO_2 compensation point in wild-type and transgenic *Arabidopsis* and tobacco plants by measuring CO_2 exchange in plants exposed to a range of CO_2 concentrations between 0 and 150 µL/L CO_2. In Table 2 we show that the average CO_2 compensation point was significantly ($P < 0.01$) lower in transgenic *Arabidopsis* and tobacco plants than in the respective wild-types. These data suggested that in both species the CO_2 concentration in close proximity to RubisCO was higher in the transgenic than in the wild-type plants. This is in agreement with the steeper initial slope of the curves relating CO_2 fixation to its concentration in the transgenic plants which express *ictB* than in the respective wild-types (Figure 2).

Activation state of RubisCO

The slope of the curves relating photosynthetic rate to intercellular CO_2 concentration (Figure 2) was steeper in the *ictB*-expressing plants than in their corresponding wild-types, suggesting an apparent higher affinity to CO_2 in the transgenic plants. This could be due to a higher level of RubisCO activity (Bainbridge *et al.*, 2000; Mott and Woodrow, 2000; Poolman *et al.*, 2000). We did not detect significant differences in the abundance of active sites of RubisCO per leaf surface area or per soluble proteins between the wild-types (tobacco and *Arabidopsis*) and their respective *ictB*-expressing plants. To examine the possibility that RubisCO activity (per active site) was higher in the transgenic plants, we exposed the neighbouring leaves of wild-types and of transgenic plants, of similar age, to identical ambient conditions of light intensity

Table 3 Rubisco activity in wild-type (WT) and transgenic tobacco plant number 3 grown in the high (70–75%) humidity chamber. RubisCO activity was determined with (*in vitro*) or without (*in vivo*) prior activation. The reaction was terminated after 1 min. Other conditions as described in Experimental procedures

Plant	RubisCO activity (nmol C fixed/nmol catalytic site/min)
WT, *in vitro*	105 ± 7
Transgenic, *in vitro*	103 ± 8
WT, *in vivo*	84 ± 7
Transgenic, *in vivo*	86 ± 6

n = 6, ± SD.

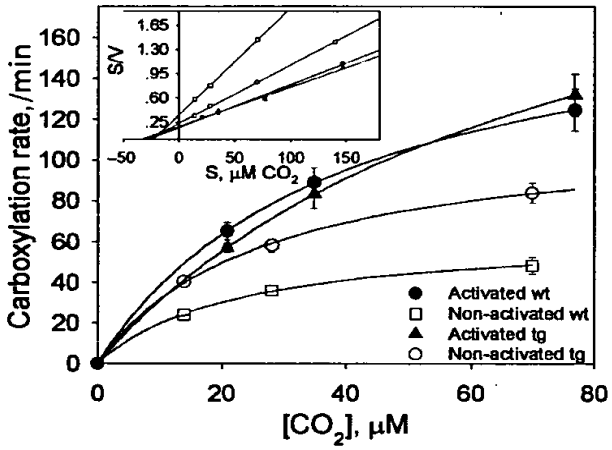

Figure 3 RubisCO activity *in vivo* (non-activated) and following *in vitro* activation in the wild-type (wt) and transgenic (tg) tobacco plant 3 grown under low humidity as affected by the CO_2 concentration during the assay. Rate of carboxylation is in nmol CO_2 fixed/nmol active sites/min. The inset provides the S/V vs. S plots of data from similar experiments. n = 6.

and orientation, temperature and relative humidity (either high or low) for several days. The leaves were excised 3 h after onset of illumination in the growth chamber and placed in liquid nitrogen (see Experimental procedures). These experiments were performed on wild-types and transgenic *Arabidopsis* and tobacco plants. As an example, we provide detailed results obtained in the experiments with the wild-type and transgenic line 3 of tobacco (Table 3, Figure 3).

Following *in vitro* activation by the addition of CO_2 and $MgCl_2$, where RubisCO activity was close to its maximum (Badger and Lorimer, 1976; Crafts-Brandner and Salvucci, 2000; Marcus and Gurevitz, 2000), there was no significant difference between the activities observed in the wild-type and transgenic plants maintained in either the humid (Table 3) or the dry conditions (Figure 3). These data confirmed that the insertion of *ictB* did not alter the intrinsic properties of RubisCO. Under the humid conditions, the RubisCO activity observed without *in vitro* activation (most likely closely resembling those *in vivo* just before the leaves were immersed in liquid nitrogen; Bainbridge *et al.*, 2000; Crafts-Brandner and Salvucci, 2000), was about 85% of that of the *in vitro* activated enzyme in both the wild-type and transgenic plants (Table 3). In contrast, under the low humidity conditions, the *in vivo* activity of RubisCO was about 40% higher in the transgenic than in the wild-type plants over the entire range of CO_2 concentrations examined in the activity assays (Figure 3). In Figure 3 we show the activities of RubisCO exposed to different CO_2 concentrations in order to emphasize the consistency of the data, even at various CO_2 levels, rather than to provide a complete account of the kinetic parameters of activated and non-activated RubisCO from tobacco. Nevertheless, an analysis of the kinetic parameters from experiments similar to that depicted in Figure 3, performed with the wild-type and transgenic line 3, indicated that while the $K_m(CO_2)$ was scarcely affected by the expression of *ictB*, the V_{max} of carboxylation, *in vivo*, was significantly higher in the *ictB*-expressing plants. The higher *in vivo* RubisCO activity in the transgenic plants as compared with the wild-type (Figure 3), under the dry conditions where stomata conductance may limit CO_2 supply, is consistent with the steeper slope of the curve relating photosynthetic rate to intercellular CO_2 concentration (Figure 2). Naturally, the *in vivo* RubisCO activities were lower than those depicted by the *in vitro* activated enzyme (Figure 3, Table 3). The reduced *in vivo* RubisCO activity in the dry vs. the high humidity grown wild-type plants is possibly due to the lower internal CO_2 concentration imposed by the decreased stomatal conductance. These are also the conditions where the transgenic plants exhibited faster photosynthesis (Figure 2) and growth (see below).

Growth experiments

In view of the enhanced photosynthesis in the transgenic plants under CO_2 limiting conditions (Figure 2), we examined how their growth was affected by the relative humidity. There was no significant difference between the growth of wild-type or transgenic *Arabidopsis* plants maintained under high (70–75%) humidity (Figure 4). Under low humidity (25–30%), both wild-type and transgenic plants grew slower than in humid conditions, but the transgenic plants grew significantly faster ($P < 0.03$) than the wild-type. In Figure 4 we provide the relative growth rates (RGR) and the dry

weight accumulated over 18 days of growth. Enhancement of growth of the transgenic plants was observed throughout the growth period and not at a particular phase. Transgenic tobacco plants that expressed *ictB* also appeared to grow faster than the wild-type under low humidity. However, due to technical limitations (the size of the plants and the need to maintain them under identical conditions within the growth chamber), detailed growth experiments with enough plants to enable statistical analyses, were only performed on *Arabidopsis*.

Discussion

Expression of a single gene from the cyanobacterium *Synechococcus* PCC 7942, *ictB*, enhanced photosynthesis and growth in C_3 plants. The increased photosynthetic rate at the limiting CO_2 level was most probably due to a higher RubisCO activity in the transgenic plants as opposed to another report where a higher level of fructose-1,6/sedoheptulose-1,7-bisphospate phosphatase raised the level of ribulose 1,5-bisphosphate and thereby stimulated photosynthesis (Miyagawa *et al.*, 2001). In the absence of an independent method for directly determining CO_2 concentration in close proximity to RubisCO, we had to rely on measurements of the CO_2 compensation point (Table 2). The lower compensation point in transgenic plants expressing *ictB* suggested that the CO_2 concentration at RubisCO sites was higher than in the wild-types, but the mechanism involved is not yet known. As indicated (Introduction), while clearly involved in Ci accumulation in *Synechococcus* PCC 7942, the role of *ictB* in Ci uptake in cyanobacteria is not yet understood. Complete inhibition of Ci transport in a *Synechocystis* sp. strain PCC 6803 mutant which possesses a normal *slr1515* (a homologue of *ictB*), suggested that *slr1515* may not be essential for Ci transport in this organism (Shibata *et al.*, 2002) which accumulates far less Ci internally than does *Synechococcus* PCC 7942. Nevertheless, it is most likely that the elevated activity of RubisCO in the transgenic plants was due to a higher CO_2 concentration that could enhance the enzyme activity, both as an activator and as a substrate. Apart from the concentration of CO_2, *in vivo* RubisCO activity is affected by several effectors and parameters including light intensity, pH, the levels of specific metabolites, magnesium concentrations and the activity of RubisCO activase (Badger and Lorimer, 1976; Bainbridge *et al.*, 2000; Cleland *et al.*, 1998; Crafts-Brandner and Salvucci, 2000; Uemura *et al.*, 1997; Harrison *et al.*, 2001; Kallis *et al.*, 2000; Spreitzer, 1999). At this time it is not known whether any of these was affected by the expression of *ictB* in the transgenic plants and thus the reason(s) for the elevated activity of their RubisCO under low humidity and limiting CO_2 is not fully understood.

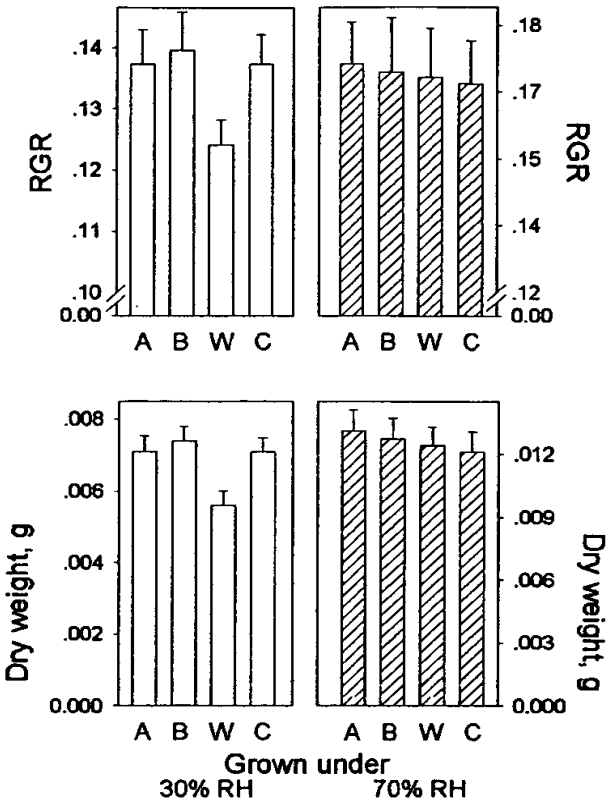

Figure 4 Growth of transgenic (A, B and C) and WT (W) *Arabidopsis* plants. Data are provided as the relative growth rate (RGR) and the average dry weight ± SD. The growth experiments were performed six times for 18 days each, $n = 18$. RH, relative humidity.

The level of *ictB* expression varied markedly between transgenic plants (Figure 1). Nevertheless, we did not detect statistically significant differences between *ictB*-expressing lines, within a given species, with respect to the CO_2 compensation point, RubisCO activity, photosynthetic performance or growth. It is possible that despite the different levels of transcription, the abundance of IctB was similar in the transgenic plants, or that a small level of IctB suffices. Our attempts to produce reliable antibodies to IctB to examine these possibilities were not successful. Those produced, directed to the hydrophilic region within this very hydrophobic protein, were not specific enough. Furthermore, in the absence of a reliable antibody, we could not examine the location of this protein in the transgenic plants and confirm that, as expected, the fusion to the transit peptide of the small subunit of RubisCO directed the protein to the chloroplast. Nevertheless, our data clearly suggested a potential use of *ictB* in raising the yield of C_3 plants, particularly under dry conditions where stomatal

closure may impose a CO_2 limitation and thus photosynthetic retardation.

Experimental procedures

Growth conditions and construction of transgenic plants

The plants used here were grown in controlled growth chambers (Binder, Germany). Tobacco (*Nicotiata tabacum*) plants were grown at 24 °C, light intensity was 350 µmol.photons/m^2/s, 12 h:12 h light : dark; *Arabidopsis thaliana* plants were grown at 21 °C, 200 µmol.photons/m^2/s, 8 h:16 h light : dark. The plants were grown in two growth chambers, the relative humidity was maintained at 25–30% in one chamber and 70–75% in the other. Transgenic tobacco and *Arabidopsis* expressing *ictB* were raised using a construct consisting of the 35S promoter fused to a DNA fragment encoding the transit peptide of the small subunit of RubisCO from pea, connected in-frame to *ictB*. This construct was inserted in *Agrobacterium* strains GV 3101 or LBA 4404 for *Arabidopsis* or tobacco transformations, respectively (Clough and Bent, 1998; Fraley *et al.*, 1985). The *Agrobacterium* vectors contained a NOS terminator and a kanamycin-resistance encoding cassette.

Measurements of photosynthetic rate and CO_2 compensation point

CO_2 and water vapour exchange were determined with the aid of a Li-Cor 6400, operated according to the manufacturer's instructions (Li-Cor, Lincoln, NE). Saturating light intensities of 750 and 500 µmol.photons/m^2/s were used during the measurements with tobacco and *Arabidopsis*, respectively. The CO_2 compensation point was deduced from measurements of the rate of CO_2 exchange as affected by a range (0–150 µmole CO_2/L) of CO_2 concentrations. The CO_2 concentration where the curve relating net CO_2 exchange to concentration crossed zero CO_2 was taken as the compensation point.

Measurements of RubisCO activity

The plants were grown for 18 days under low or high relative humidity with temperature and light conditions as above. They were placed at a similar distance and orientation from the light sources to minimize possible differences between them due to unequal local conditions. The leaves were excised 3 h after the onset of illumination and immersed immediately in liquid nitrogen. Fifteen cm^2 of frozen leaves were ground in a buffer containing 1.5% PVP, 0.1% BSA, 1 mM DTT, protease inhibitors (Sigma) and 50 mM Hepes-NaOH pH 8.0. For *in vitro* activation, the extracts were centrifuged and aliquots of the supernatants were supplemented with 10 mM $NaHCO_3$ and 5 mM $MgCl_2$ (Badger and Lorimer, 1976) and maintained for at least 20 min at 25 °C. RubisCO activity was determined, either immediately or after the activation (Marcus and Gurevitz, 2000) in the presence of 20–150 µM $^{14}CO_2$ (6.2–9.3 Bq/nmole). The reaction was terminated after 1 min by 6 N acetic acid and the acid-stable products were counted in a scintillation counter (Marcus and Gurevitz, 2000). Time course analyses indicated that the RubisCO activities were constant for 1 min and declined thereafter, probably due to the accumulation of inhibitory intermediate metabolites (Cleland *et al.*, 1998; Edmondson *et al.*, 1990; Kane *et al.*, 1998). Quantification of the amount of RubisCO active sites was performed as in Marcus and Gurevitz (2000).

Growth experiments

Wild-type and transgenic *Arabidopsis* plants were germinated and maintained for 10 days under humid conditions. To minimize possible variations in water supply between transgenic and wild-type plants, the seedlings were transferred to pots, each containing a wild-type and three different transgenic plants. Twelve pots were placed in each growth chamber (Binder, Germany) under equal light intensity and temperature. The relative humidity was maintained at 25–30% in one chamber and 70–75% in the other. Other growth conditions were as above. The plants were harvested after 18 days of growth, quickly weighed (fresh weight) and dried in the oven (dry weight). The growth experiments were repeated six times.

RNA isolation

For each sample, 1–2 g of plant material were ground in liquid nitrogen using a mortar and pestle and then transferred to 5 mL of Tris-HCl buffer (50 mM Tris-HCl, pH 8, 300 mM NaCl, 5 mM EDTA, 2% SDS, 2 mM Aurin tricarboxylic acid (ATA) and 14 mM β-mercaptoethanol) at room temperature, vortexed, and incubated on ice for 10 min. After adding 0.7 mL of ice-cold 3 M KCl the homogenates were vortexed and incubated on ice for 15 min. They were then centrifuged in a Sorvall SS-34 rotor at 10 000 r.p.m. for 10 min at 4 °C. The supernatants were transferred to new tubes containing 2 mL of 8 M LiCl and well mixed. The samples were incubated overnight at 4 °C and then centrifuged as described above.

The supernatants were discarded and the tubes were left upside down to drain for two min. The pellets were dissolved by vortexing in 0.4 mL of DEPC-treated water containing 0.5 mM ATA.

The RNA from each sample was extracted with 0.6 neutralized phenol in microfuge tubes and the upper aqueous phase was transferred to a new sterile tube containing 0.1 volume of 3 M Na-acetate and vortexed. Two volumes of cold ethanol were added, vortexed and incubated at –20 °C for 2 h. The samples were centrifuged for 30 min at 16 000 ***g*** in a microfuge. The pellets were rinsed with 1 mL cold 80% ethanol, centrifuged 10 min at 14000 r.p.m. and then the supernatants were discarded. The pellets were air-dried for 5 min and then resuspended in 25 µL sterile water containing 0.5 mM ATA.

Acknowledgements

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